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Universidad del País Vasco

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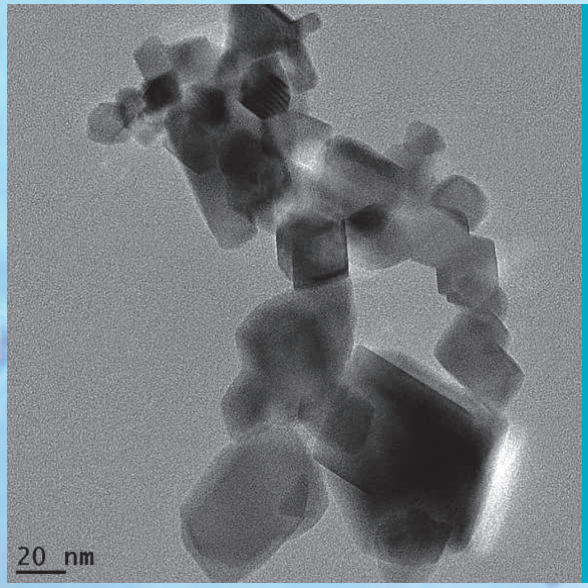
Towards the standardization of nanoecotoxicity testing:

Selection of environmentally relevant methods



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Doctoral Thesis

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Universidad del País Vasco
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Towards the standardization of nanoecotoxicity testing:

Selection of environmentally relevant methods

Submitted for the Doctoral degree certificate at the University of the Basque Country (UPV/EHU)

by

Cristina Cerrillo Redondo

Leioa, February 2016

Supervisors:
Dr. Gotzone Barandika Argoitia
Dr. Amaya Igartua Aranzábal

NANOREG
SEVENTH FRAMEWORK PROGRAMME

IK4 TEKNIKER
Research Alliance

A mi familia

A todos los que están a mi alrededor

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Allí estaba otra vez, la verdad incontestable. Pablo casi cayó muerto ante la revelación de la Vida. Pero su corazón siguió latiendo. Paradojas que tiene la gran ballena blanca.

-Pero no acabo de comprenderlo. Algo está fuera de lugar. Todos esos hombres que te buscaron con ardor, que se midieron contigo, están ahora muertos ¿Cómo es posible que la vida los matara?

-Ése es el gran misterio –suspiró Moby Dick- pero creo que puedo aclarártelo. Murieron porque, cuando me encontraron, no me comprendieron, no desentrañaron el significado que llevo escrito. Cuando me tuvieron frente a frente, decidieron no seguir viviendo. Dejaron de buscar. Y la vida por sí sola no sobrevive. Si la búsqueda de los hombres no la alimenta, termina por tener hambre y los devora. El capitán Ahab, Jonás... se dejaron arrastrar, se abandonaron a mi voracidad sin fin. No entendieron, cuando me hallaron, que no era yo quien los hacía estar vivos, sino que eran ellos los que me sostenían a mí. No comprendieron que hay que estar a la altura de la vida, que tenían que enfrentarme y seguir luchando, por eso no les quedó otra salida que la muerte. Ya no estaban hechos para mí ni yo para ellos.

Pablo se estremeció ante aquella crudeza desnuda e insoslayable.

-¿Y por qué yo todavía estoy vivo, hablando en tu vientre?

-Porque tú aún te mereces la vida. Porque tú aún tienes derecho a acogerte en lo más profundo de mi seno.

-¿Y por qué? ¿En qué me diferencio yo de todos los demás, de todos los que ahora están muertos?- Inquirió Pablo.

-En que tú aún sigues buscando, en que todavía tienes algo que hacer en este mundo. Tú no te hundes conmigo en las profundidades innominadas, te sobrepones a la misma vida porque puedes seguirla. Eres la gota que vuela con mi impulso, dispuesta a alcanzar las estrellas, a escalar hasta el firmamento, a las regiones adonde perteneces, donde está tu hogar, donde lucen tus sueños, donde trazar tu propio camino, donde conocer tu verdad. Eso es cuanto puedo desvelarte. El resto tendrás que averiguarlo tú. Tendrás que descubrir qué es aquello en lo que me encuentres de una forma especial y única: allí estará tu verdad. Esa será tu propia vida.

-¿Y ya no me vas a ayudar para que averigüe qué es y en qué está?

-Por supuesto que sí. Vas a seguir viviendo, ¿te parece poco? Con eso te lo doy todo... pero no lo olvides, en cualquier momento podrías volver a toparte conmigo y, esa vez, ser acreedor de la muerte. Sigue buscando, Pablo. Y recuerda que, el día que descubras, descubrirás también que debes seguir buscando. Esa es la única forma de vivir. Hasta la vista. Me encontrarás en todo y, lo más importante, me encontrarás en ti y en todos tus pasos. Para encontrarme, sigue andando.

"Dime una palabra" – Marta Quintín Maza

The research presented herein has been written following the contributions format. It includes a general summary, an introduction dealing with the current state of knowledge of the research area, the hypothesis proposed, aim of the work, methodologies used, a general overview of the obtained results, the publications derived from the study conducted, conclusions, future perspectives and a detailed description of the instrumental techniques utilized. The CD version of the work is attached to this printed document.

Summary

Nanotechnology, the science of manipulating the physicochemical properties of materials on the atomic and molecular level, provides innovative solutions to various scientific disciplines. The potential and real applications of manufactured nanomaterials (MNMs) in physics, chemistry, information technology, or medicine are growing exponentially. However, their novel features have led to questions about physical, health and environmental risks.

Natural resources and biodiversity constitute fundamental assets for the survival of all kind of live in the Earth. One of the most important pathways for the entrance of MNMs and their transfer throughout the food web is represented by aquatic organisms, but the lack of standardized assessment protocols has led to contradictory toxicity results in natural waters. Certain aspects, namely, sample preparation and characterization, dosimetry, exposure data and model organisms, require further validation and regulation.

The present study was aimed at defining test methods to overcome the limitations of the toxicological assessment of MNMs in aquatic ecosystems. Organisms of different trophic levels, selected in terms of cost, ecological relevance, reproducibility and sensitivity, were tested (seawater bacterium *V. fischeri* and the freshwater crustacean *D. magna* and microalgae *P. subcapitata*). Multiwalled carbon nanotubes (MWCNTs) and TiO_2 and CeO_2 nanoparticles, currently included in the prioritization lists of relevant reference materials worldwide, were the MNMs subjected to analysis.

The standardization approach of the current work was addressed through the optimization of the energy delivered to the MNMs during the preparation of the aqueous dispersions, and the selection of a reference natural organic matter to conduct the exposures in environmentally realistic conditions. The methodologies proposed have improved the reproducibility of the toxicity test results. In addition, the influence of the test materials and methods in the colloidal stability of MNMs and their adverse effects towards aquatic organisms has been demonstrated.

The EU-FP7 NANoREG project, aimed to give an answer to regulators and legislators on Environmental, Health and Safety aspects of MNMs, has provided an essential framework for the present study.

Abbreviations

a.u.	Arbitrary units
CL	Confidence limits
CNTs	Carbon nanotubes
DLS	Dynamic Light Scattering
EC	European Comission
EC10, EC20, EC50	Effective concentrations causing 10%, 20% and 50% inhibition, respectively
EHS	Environment, Health and Safety
ENMs	Engineered nanomaterials
EPM	Electrophoretic mobility
HA	Humic acid
ISO	International Organization for Standardization
JRC	Joint Research Centre-European Commission
MNMs	Manufactured nanomaterials
MWCNTs	Multiwalled carbon nanotubes
NOM	Natural organic matter
NPs	Nanoparticles
OECD	Organisation for Economic Co-operation and Development
PDI	Polydispersity index
QNAR	Quantitative nanostructure-activity relationship
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
SEM	Scanning Electron Microscopy
SR-NOM	Suwannee River natural organic matter
SWCNTs	Single-walled carbon nanotubes
UV/Vis	Ultraviolet-visible
UV/Vis/NIR	Ultraviolet-visible-near infrared
Z_{ave}	Zeta-average diameter

Contents

Chapter 1. Introduction

1.1. Materials Science and Technology: time and size evolution

Human beings have developed and exploited materials from the beginning of their history to meet their needs. Major technological advances, namely the steam-engine or modern telecommunications, have influenced positively the welfare of humankind. The quality of life and life expectancy achieved at present may be attributed mainly to the progress made in the multidisciplinary field of Materials Science and Technology. This discipline is concerned basically with investigating the physicochemical aspects of matter to provide practical improvements in many diverse sectors, such as construction or medicine. Nowadays, multiple new materials are constantly being devised, manufactured and optimized for applications in almost every domain of industrial activity. Some nations have strategically invested in new technologies, such as South Korea and Taiwan, which have become world leaders in high-tech manufacturing. This innovation process has enabled greater

access to new markets for developing countries, resulting in improved knowledge and acting as a further catalyst for economic growth. Therefore, recent applications of materials are considered capable of helping fight global inequality.[1]

The microtechnology of the second half of the 20th century gave rise to a technical revolution that led to the extensive manufacturing of computers and the advent of the Internet, taking us into the dynamic emerging era of nanotechnology.[2] This reality was predicted by Richard Feynman in 1959. In his speech "There's plenty of room at the bottom" at the annual meeting of the American Physical Society,[3] Feynman foresaw the possibility of manipulating and controlling matter on a small scale, and visualized printing all 24 volumes of the Encyclopedia Britannica on the head of a pin. Fifty years after computers occupied entire floors of buildings, materials and devices can be controlled on the atomic and molecular level (Figure 1.1). Nanotechnology became an established discipline of scientific investigation and engineering with the invention of the scanning tunnelling microscope (STM) in 1981, and has achieved major breakthroughs in its short history.[4] It can be defined as the manipulation, precision placement, measurement, modelling or manufacturing of materials at the sub-100 nanometer scale, where the size dependent modulation in its physicochemical properties leads to novel functionalities. While Feynman is credited with heralding its coming, Eric Drexler is credited with coining the term *nanotechnology* in the 1980s.[5]

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[1] Guillaume Flament. Closing the Gap: The Impact of Nanotechnologies on the global Divide. Nanotechnology Industries Association (NIA) Report. Bruxelles, Belgique. 2013.

[2] Dreher KL. **2003**. Health and Environmental Impact of Nanotechnology: Toxicological Assessment of Manufactured Nanoparticles. *Toxicol Sci* 77:3-5.

[3] Feynman R. There's plenty of room at the bottom. Caltech Engineering & Science. 23:22-36.1960.

[4] Kumar A, Dhawan A. Manual on Critical Issues in Nanotechnology R&D Management-An Asia-Pacific Perspective. Chapter 1: Nano-safety, standardization and certification. 2013.

[5] Klaine SJ, Koelmans AA, Horne N, Carley S, Handy RD, Kapustka L, Nowack B, Kammer F. **2012**. Paradigms to assess the environmental impact of manufactured nanomaterials. *Environ Toxicol Chem* 31:3-14.

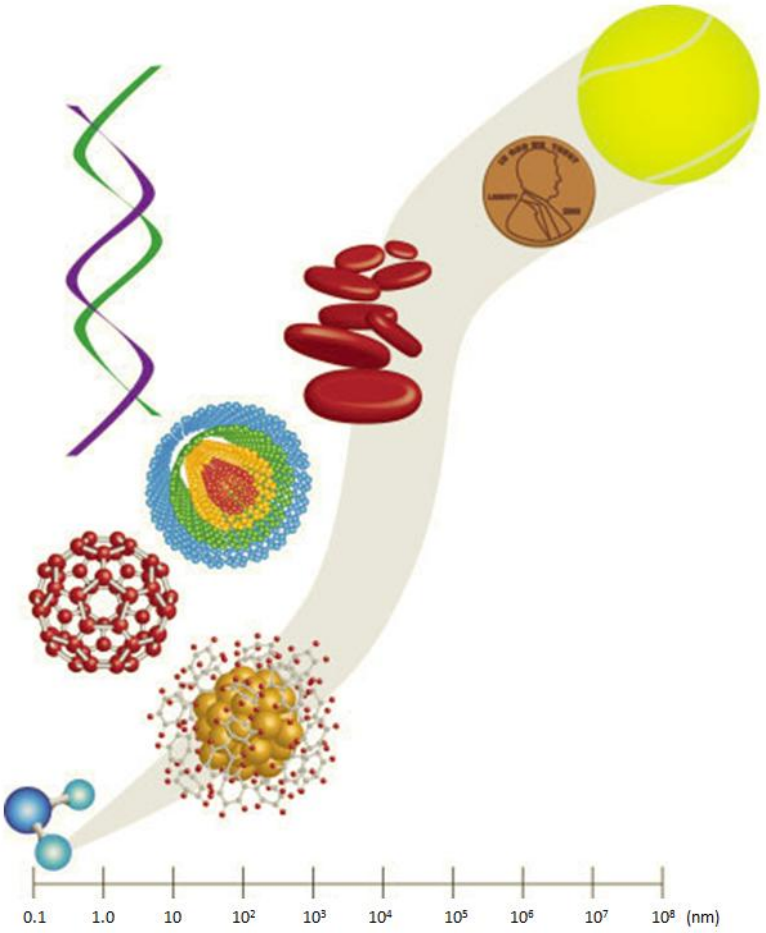

Figure 1.1 Tipping the scale: The relative size of materials in our world. Left to right: Water molecule (H_20); Citrate stabilized gold sphere; Buckminster fullerene (C60); DNA; multiwalled carbon nanotube; red blood cells; penny; tennis ball.[5]

Since then, the increasing sophistication of nanotechnologies has been supported by the significant progress in microscopy and other analysis tools, the advances in nanomaterial synthesis, and funding from numerous organizations. The effective visualization, design and control of matter are enabled by the understanding of its behavior at the nanoscale, according to the laws of quantum physics. Furthermore, the size reduction leads to increased surface area imparting new physicochemical properties. Nanotechnology provides innovative solutions to various scientific disciplines, such as physics, chemistry, information technology, medicine and biology. Considering that applications can be found in almost every domain of industrial activity, it is experiencing a great impact on the world economies. Although industrialized countries are leading the global race for nanotechnologies, developing countries could also find applications to boost their economic growth, and many achievements are closer to commercialization. The access to clean water, medical solutions, diagnosis and treatment, agricultural crop optimization and

energy production and storage are target areas for the innovative solutions supported by the use of nanotechnologies.[4]

There are numerous definitions of the term nanomaterial, although they all concur that they are materials whose size or structure has at least one dimension measuring between 1 and 100 nanometers.[6] Nanomaterials (NMs) may present very different structure and composition, and include not only particulate materials, but also nano-structured volume- or surface domains. The Organisation for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO) are the most prominent organizations working on schemes for defining and classifying nanomaterials, which constitute key tasks for their legislation. In accordance with the Core Terms of ISO, nanomaterials are defined as so-called nano-objects or nanostructured materials. This definition includes particle-like, rodlike or platelet-shaped objects and their assemblages, the generic term for agglomerates and aggregates. All three dimensions are less than 100 nm in the case of nanoparticles (NPs). Agglomerates are considered as weakly bound collection of interconnected NPs, whereas strongly bound particles form aggregates.[7] Some of these morphologies are shown in Figure 1.2.

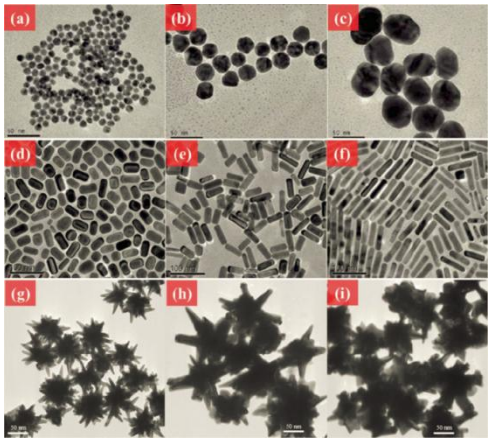

Figure 1.2 TEM images of Au NMs. (a–c) Au NPs in different sizes. (d–f) cetyltrimethyl ammonium bromide-coated Au nanorods with different aspect ratios. (g–i) Au nanourchin with different morphologies.[8]

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[6] Assessment of the risks associated with nanomaterials, issues and update of current knowledge. French Agency for Food, Environmental and Occupational Health & Safety (ANSES). May 2014.

[7] Lang J, Meyer-Plath A. Characterisation of substances at nanoscale as background for the regulation in the framework of the regulation (EC) No. 1907/2006 (REACH). Federal Institute for Materials Research and Testing (BAM), Berlin, Germany. 2013.

[8] Cheng L-C, Jiang X, Wang J, Chen C, Liu R-S. **2013**. Nano-bio effects: interaction of nanomaterials with cells. *Nanoscale* 5:3547-3569.

Since time immemorial, organisms and the environment have been exposed to natural nanoparticles like volcanic dust, ash, organic matter like humic and fulvic acids, proteins, peptides and colloidal inorganic species present in natural water and in soil systems.[9] Incidental nanoparticles, also defined as anthropogenic nanoparticle waste, are produced as a result of human activities such as industrial processes, coal combustion, welding fumes, or vehicle exhaust (e.g., carbon black, soot). In contrast to nascent and incidental nanoparticles, manufactured or engineered nanomaterials (MNMs, ENMs) are designed to exhibit specific physicochemical properties targeted towards unique applications[10] (Figure 1.3).

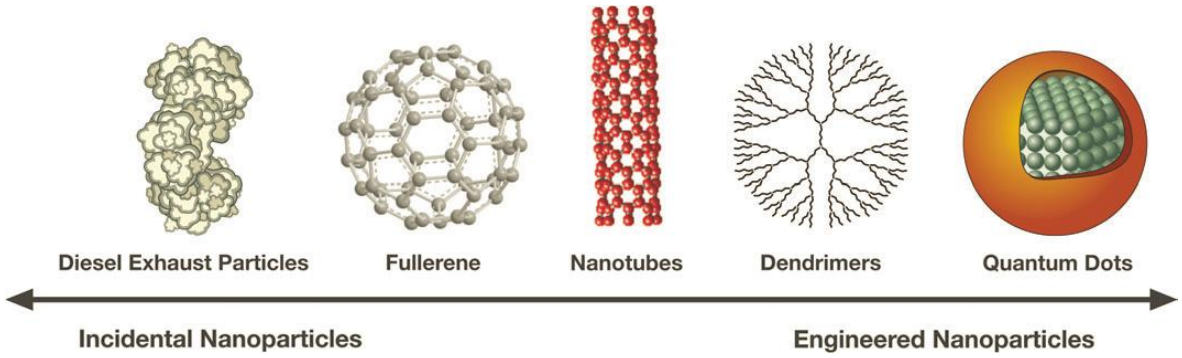


Figure 1.3 Examples of incidental and engineered nanoparticles.[11]

In 2012, the global production of MNMs was devoted mainly to carbon black, synthetic amorphous silica, aluminum oxide, barium titanate, titanium dioxide, cerium oxide, zinc oxide and silver.[12] They were used for commodity applications in everyday goods, and also in highly specialized low-volume technical applications, e.g. in electronics or biomedicine. Nonetheless, a thorough analysis to estimate the number of products containing nanomaterials is not feasible. It was reported to be between 500 and 2000 in 2014.[13] Moreover, the estimates of production volumes

[9] Soni D, Naoghare PK, Saravanadevi S, Pandey RA. Release, Transport and Toxicity of Engineered Nanoparticles. Whitacre DM (ed.), *Rev Environ Contam T*, Vol 234. Springer International Publishing, Switzerland, pp 1-48. 2015.

[10] Yadav T, Mungray AA, Mungray AK. Fabricated Nanoparticles: Current Status and Potential Phytotoxic Threats. Whitacre DM (ed.), *Rev Environ Contam T*, Vol 230. Springer International Publishing, Switzerland, pp 83-110. 2014.

[11] Stern ST, McNeil SE. **2008**. Nanotechnology safety concerns revisited. *Toxicol Sci* 101:4-21.

[12] Hartl S, Fries R, Giovanna DD, Klein J, Micheletti C, Laganis J, Łojkowski W, Sobczyk J, Swiderska-Sroda A, Falk A. "NANOFORCE" Nanotechnology for Chemical Enterprises-how to link scientific knowledge to the business in the Central Europe space. Book of recommendations for the European Commission-Longversion. 2013.

[13] Hermann A, Diesner M-O, Abel J, Hawthorne C, Greßmann A. Assessment of Impacts of a European Register of Products Containing Nanomaterials. Federal Environment Agency, Germany. 2014.

available often differ considerably from one another. A comprehensive study based on several years indicated that SiO_2 and TiO_2 were the nanomaterials with the highest production volumes worldwide and probably the most common in products as well[14] (Table 1.1).

Table 1.1 Estimated global production volumes of different nanomaterials.[14]

Nanomaterial	Production volumes (in tons p.a.)	Year
SiO_2	1590000	2009
TiO_2	700-61000	2007/2008
	50000	2010
	44000 (only USA)	2008
	1450 (only Japan)	2019
ZnO	20-10000	2007/2008
	480 (only Japan)	2009
CeO_2	10000	2010
Al oxides	100	2003
ZrO_2	2500	2010
Silver	4-560	2005/2008
Quantum dots	< 100 kg	2001
Nanoclays	9000	2007
Carbon Nanotubes	140-150	2004/2006–2008
	120-140	2009
	750 (approximately)	2011
Graphene	15	2007
Fullerenes	0.2-10	2002/2005/2008
	3	2009

The current and various applications of MNMs can be classified in product categories, namely: textiles, cosmetics, paints and varnishes, building materials,

[14] Greßler S, Part F, Gazsó A. "Nanowaste" - Nanomaterial-containing products at the end of their life cycle. NanoTrust Dossier No. 040en - August 2014. Institute of Technology Assessment of the Austrian Academy of Sciences. 2014.

sports equipment and electronics industry.[14] A detailed list of applications and products is included in Table 1.2.

Table 1.2 Commonly used manufactured nanomaterials and their applications.[15,16]

Nanomaterial	Producs/Applications
Carbon nanotubes (CNTs)	Electronics and computers, batteries, supercapacitors, adhesives and composites, biological and medical applications, sensors, orthopedic implants, sporting goods, adsorbents for the removal of pollutants from water.
Fullerenes (C_{60})	Optical and electronic devices, superconductors, sensors, catalysts, polymer modifications, lubricants, sporting goods, drug delivery, cosmetics.
Carbon nanohorns	Catalyst supports and drug delivery.
Carbon nanowires	Early detection of cancerous tumor.
SiO_2 NPs	Pharmaceutical products, vegetable oil refining, detergents, adhesives, fire proof glass, ceramics, fillers, electronics, chromatography, catalysts.
SnO_2 NPs	Transparent conducting coating of glass, gas sensors, solar cells, heat mirrors, catalyst supports.
TiO_2 NPs	Food coloring, paints, self-cleaning window coatings, fillers, photocatalysts, bioremediation (removal of various organics), additive in pharmaceuticals and cosmetics, UV-protection, sunscreen lotions, antibacterial agent.
ZnO NPs	Electrical and optical devices, electrostatic dissipative coating, semiconductors, chemical sensors and solar cells paints, diode lasers, chemical absorbent, catalysts, sunscreens, cosmetics, UV-protection.
Al_2O_3 NPs	Batteries, catalysts, adsorbent, grinding, polishing abrasives.
CeO_2 NPs	Combustion catalyst in diesel fuels to improve emission quality, gas sensors, solar cells, oxygen pumps, metallurgical and glass/ceramic applications, semiconductor devices.
Fe_3O_4 NPs	Removal of contaminants, sensors, magnetic storage media, magnetic refrigeration, magnetic resonance imaging (MRI), DNA detection, drug delivery system, cancer therapy.
Magnesium–aluminum oxide, $MgAl_2O_4$ NPs	Sensors, catalysts.
Zero-valent iron (Fe^0) NPs	Remediation of water, sediments, and soils to remove nitrates and organic contaminants.
Ag NPs	Antibacterial agent in wound dressings, socks and other textiles, toothpastes, dental resin composites, baby-products, coatings of medical equipments, vacuum cleaners, air filters, washing machines, paints.
Au NPs	Drug delivery, vector in tumor therapy, catalysts, flexible conducting inks and films.

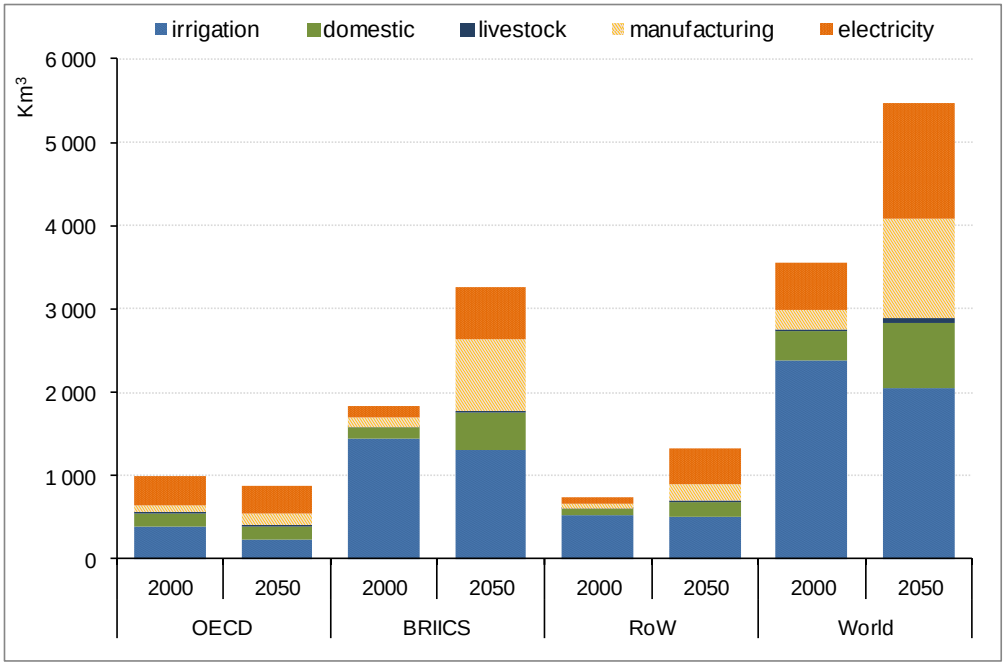
[15] Bhatt I, Tripathi BN. **2011**. Interaction of engineered nanoparticles with various components of the environment and possible strategies for their risk assessment. *Chemosphere* 82:308-317.

[16] Srivastava V, Gusain D, Sharma YC. **2015**. A critical review on the toxicity of some widely used engineered nanoparticles. *Ind Eng Chem Res* 54: 6209-6233.

Cu NPs	Catalysts.
CdS NPs	Photodetectors, optoelectronics, solar cells.
ZnS NPs	Electroluminescent devices, solar cells, phosphors.
Quantum dots (QDs)	Medical imaging and targeted therapeutics, solar cells, photovoltaic cells, security inks, photonics and telecommunications.
Dendrimers	Macrocapsules, nanolatex, coloured glasses, chemical sensors, modified electrodes, DNA transfecting agents, therapeutic agents, drug delivery, DNA chips, tumor treatment.

1.2. Key challenges for nanotechnology

The unprecedented growth and prosperity experienced by the humanity over the past four decades has been emphasized by many organizations and reports, such as the *OECD Environmental Outlook to 2050*.[17] The size of the world economy has been tripled, and population has increased by over 3 billion people since 1970, which has been accompanied by environmental degradation and natural resource depletion. According to this *Outlook*, the Earth's population is expected to increase to over 9 billion by 2050, challenging the management and restoration of the natural assets on which all life depends (Figure 1.4). Its main conclusion is that urgent and holistic action is needed to avoid significant costs and consequences of inaction, both in economic and human terms.



BRIICS=Brazil, Russia, India, Indonesia, China, South Africa. RoW=Rest of the world.

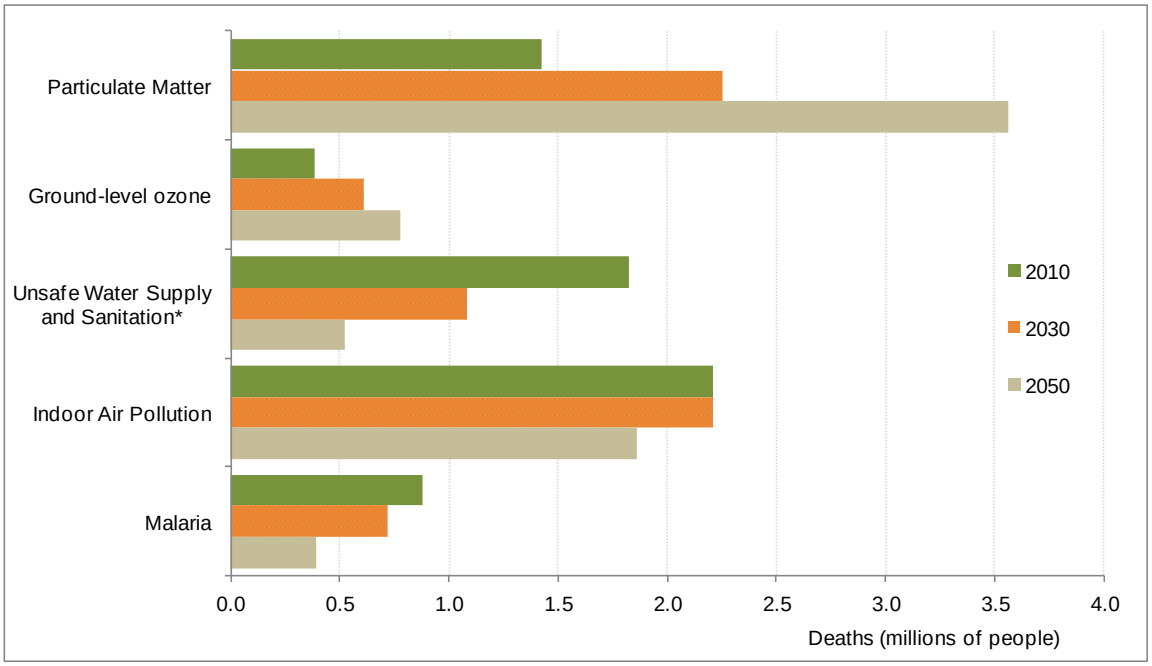
Figure 1.4 Global water demand.[18]

Without more ambitious policies, by 2050 more disruptive climate change is likely to occur, biodiversity loss is projected to continue, freshwater availability will be further strained, and air pollution is set to become the world's top environmental cause of

[17] *OECD Environmental Outlook to 2050. The Consequences of Inaction*, OECD Publishing, 2012. DOI: 10.1787/9789264122246-en

[18] *OECD Environmental Outlook to 2050. The Consequences of Inaction.* http://www.oecd.org/env/indicators-modelling-outlooks/waterchapteroftheoecdenvironmentaloutlookto2050theconsequencesofinaction.htm Last access: September 2015

premature mortality (Figure 1.5). Natural systems have inflection points beyond which damaging change becomes irreversible, and acting now is environmentally and economically rational.



*Child mortality only.

Figure 1.5 Global premature deaths from selected environmental risks.[19]

Furthermore, the different environmental concerns are closely linked. For instance, climate change can affect hydrological cycles and exacerbate pressures on biodiversity and human health. Biodiversity and ecosystem services are intimately linked to water, climate and human health: marshlands purify water, mangroves protect against coastal flooding, forests contribute to climate regulation and genetic diversity provides for pharmaceutical discoveries.

Well-designed policies can reverse the mentioned trends. Some common approaches proposed in the *Outlook* scenario were the development of effective regulations and standards to safeguard human and environmental integrity, and the encouragement of green innovation by investing in public support for basic R&D. In addition, better information was proposed to support better policies. The economic valuation to understand the benefits of biodiversity and ecosystem services, and

[19] *OECD Environmental Outlook to 2050. The Consequences of Inaction.* http://www.oecd.org/env/indicators-modelling-outlooks/healthenvchapter-environmentaloutlookto2050.htm
Last access: September 2015

health costs associated with exposure to chemicals will help to measure those elements of human welfare and progress that cannot be captured by Gross Domestic Product (GDP) alone.

Nano-technological products, processes and applications are expected to contribute significantly to environmental and climate protection. The special physicochemical properties exhibited by MNMs make them interesting for environmentally friendly products. Some of them are already available and certain applications have been implemented, but much is currently in the research and development stage. The main environmental contributions of nanotechnology are expected especially in the fields of (1) reduced use of raw materials through miniaturization, (2) energy savings through weight reduction or optimized function, (3) energy and environmental technology, (4) replacement of hazardous materials and (5) energy and resource efficiency in the chemical industry.[20] Nonetheless, the production of MNMs today often requires large amounts of energy, water and environmentally problematic chemical products such as solvents, which might hinder any potential advantages. In addition, comprehensive life cycle analyses are necessary to evaluate their real environmental effects (advantages and risks), but they are not available in most cases.

The benefits of nanoscience might be fully realized in the area of environmental technology. In recent years, the use of MNMs for environmental remediation in polluted soil, water and gas has attracted considerable attention. Various publications have demonstrated the usefulness of inorganic NPs on wastewater treatments for the removal of metals and elimination of nutrients.[21] Their characteristic high surface area, photosensitivity, catalytic and antimicrobial activity, electrochemical, optical, and magnetic properties, tunable pore size and surface chemistry are the key properties for these applications. MNMs are suitable for nanotechnology-enabled multifunctional processes capable of performing multiple tasks (e.g., water disinfection, decontamination, and separation) in one reactor. NMs of different functions can be easily assembled together, and membranes are a good

[20] Greßler S, Nentwich M. "Nano and the environment- Part I: Potential environmental benefits and sustainability effects. NanoTrust Dossier No. 026en - March 2012. Institute of Technology Assessment of the Austrian Academy of Sciences. 2012.

[21] Sánchez A, Recillas S, Font X, Casals E, González E, Puntes V. **2011**. Ecotoxicity of, and remediation with, engineered inorganic nanoparticles in the environment. *Trac-Trend Anal Chem* 30:507-516.

and extensively studied platform for these multifunctional devices (Figure 1.6).[22] The high-efficiency processes enabled by nanotechnology provide a promising route to retrofit aging infrastructure and to develop high-performance, low-maintenance, decentralized, treatment systems including point-of-use devices.

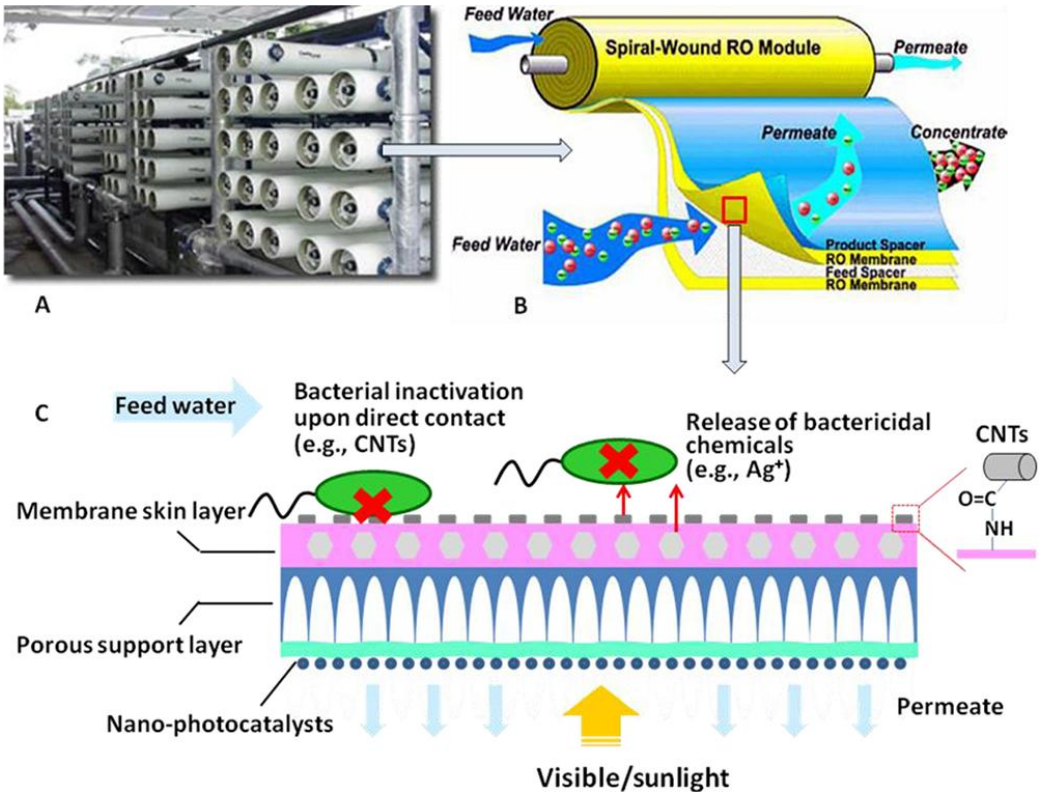


Figure 1.6 Nanotechnology-enabled multifunctional membrane system. (A) Reverse osmosis (RO) membrane water treatment system; (B) spiral-wound RO membrane module; (C) conceptual multifunctional membrane.[22]

However, the vast majority of these applications are in the stage of laboratory research with exceptions that are being field tested, and their implementation in water treatment will require overcoming the relatively high costs of MNMs. The number of publications on these topics has grown exponentially in recent years, but it has not been accompanied by knowledge about the long-term, and sometimes even the short-term behavior of nanomaterials once used and released into the environment.

The physicochemical properties that appear at the nanoscale have led to questions about potential physical (fire, explosion), health (large surface area, strong ability to

[22] Qu X, Brame J, LI Q, Alvarez PJJ. **2013**. Nanotechnology for a Safe and Sustainable Water Supply: Enabling Integrated Water Treatment and Reuse. *Accounts Chem Res* 46:834-843.

cross physiological barriers, interactions with biomolecules) and environmental risks (persistence in the environment).[6] The uncertainties on their potential health impact might lead to polarized public debate and to business unwillingness to invest further in nanotechnology. It is therefore important to ensure that an appropriate regulatory framework takes these issues into consideration.[12] Although toxicity studies on humans suggest that many NMs have the potential to induce oxidative stress and inflammation, and some may even induce granulomas, fibroses and tumours, little is known whether the effects seen in laboratory experiments are relevant at exposure levels[11] (Figure 1.7). Uncertainties also exist regarding the environmental fate and behavior of nanomaterials, mainly related to the difficulty to measure them in environmental media.[23]

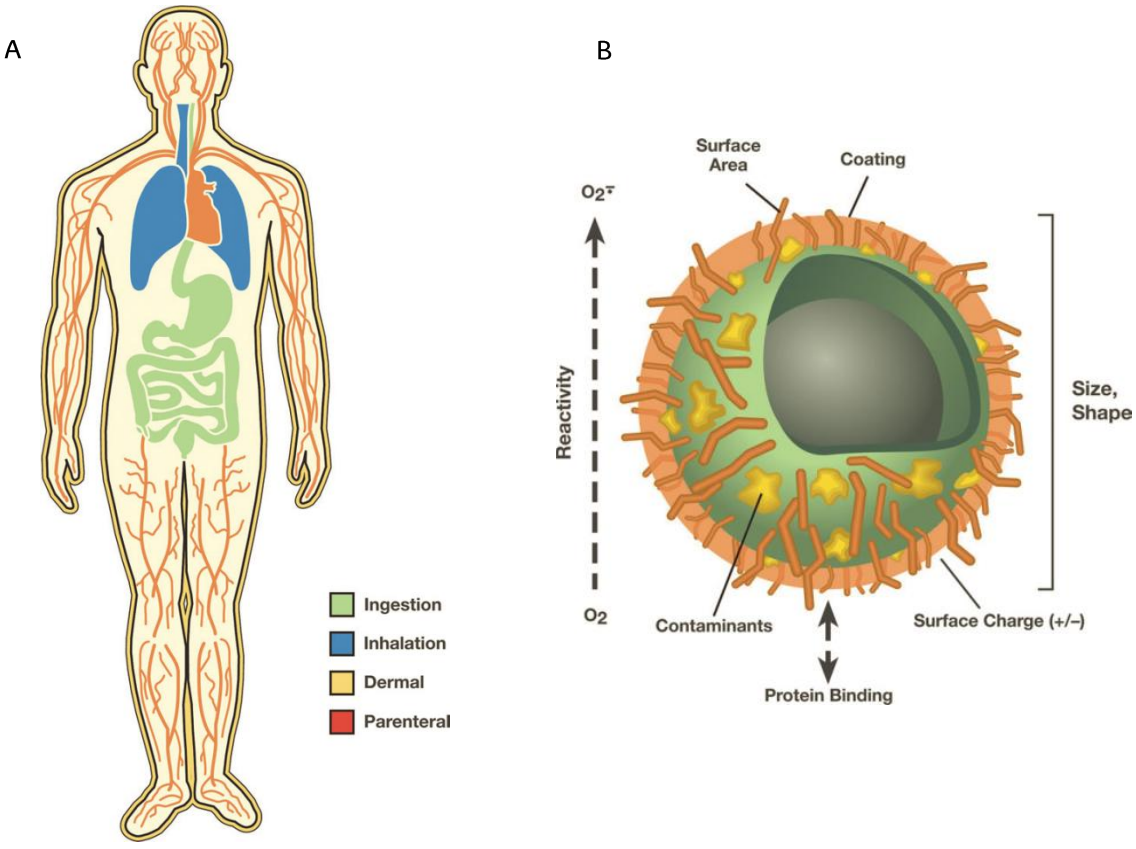


Figure 1.7 Potential routes of human exposure to nanomaterials (A); Physicochemical properties of nanoparticles that may influence biocompatibility (B).[11]

[23] Aschberger K, Rauscher H, Crutzen H, Rasmussen K, Christensen FM, Sokull-Klüttgen B, Stamm H. Considerations on information needs for nanomaterials in consumer products. Discussion of a labelling and reporting scheme for nanomaterials in consumer products in the EU. Joint Research Centre (JRC) Science and Policy Reports. European Comission. April 2014.

Nonetheless, the available information on this issue suggests that a considerable proportion of MNMs might be released to different environmental compartments. Keller et al.[24] combined market information and material flow modeling to assess the likely release of the top ten most produced ENMs for their major applications (Figure 1.8). They estimated the emissions during the manufacturing, use, and disposal stages, including intermediate steps through wastewater treatment plants and waste incineration plants. According to their study, 63-91% of over 260000-309000 metric tons of global ENM production in 2010 ended up in landfills, with the balance released into soils (8-28%), water bodies (0.4-7%), and atmosphere (0.1-1.5%).

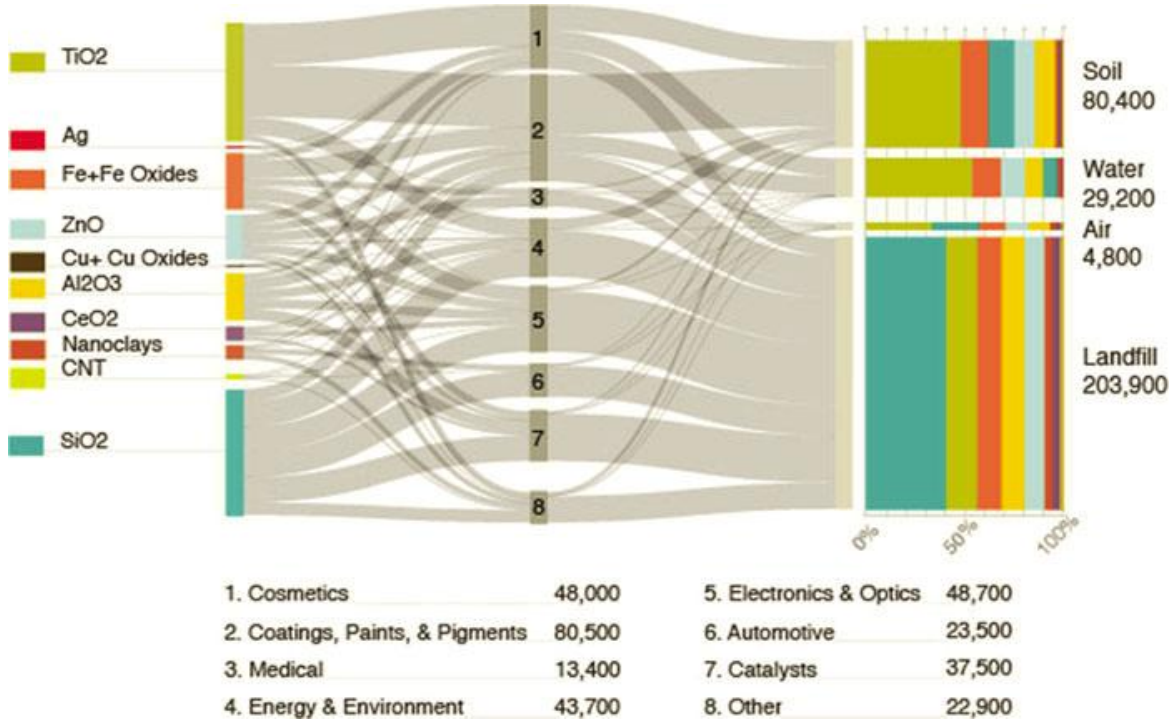


Figure 1.8 Global flow of ENMs in 2010 (metric tons/year) from manufacturing to applications and eventual disposal or release into the environment, considering the high range of production estimates and releases. Life cycle stages from left (production of ENMs) to right (final disposal or release).[24]

In addition, with the increasing number of studies of MNMs and their behavior, fate and effects, it has become clear that they are unlikely to behave like other contaminants in the environment.[25] Several investigations have shown a certain hazard potential of some nanomaterials, and even though scientific uncertainties

[24] Keller AA, McFerran S, Lazareva A, Suh S. **2013**. Global life cycle releases of engineered nanomaterials. *J Nanopart Res* 15:1692.

[25] Bour A, Mouchet F, Silvestre J, Gauthier L, Pinelli E. **2015**. Environmentally relevant approaches to assess nanoparticles ecotoxicity: a review. *J Hazard Mater* 283:764-777.

still exist, the precautionary principle should be applied in the sense of preventive risk minimization.[26]

There is a consensus among nanosafety experts that the complex interactions of nanoparticles with the environment have just started to be understood. Sometimes the new technologies present unforeseen consequences, which can be avoided if they are developed in a responsible manner. Professor Richard Owen[27] declared in a specialized documentary[28] that this is the main challenge faced by nanotechnology. He proposed that many of the questions are not raised on a technical or safety basis, but they are rather philosophical: Are the technologies that we want safe? Should we consult with public opinion? Do they improve our lives? Do they have unexpected consequences? Do they create inequality? Professors Mark R. Wiesner[29] and Cole Matson[30] also supported this approach based on dimensions of anticipation, reflection, deliberation and responsiveness. It is necessary to think about the world where these technologies are developed and the life we build around them. Over time, unforeseen impacts not detected in the experiments might appear. Although it is not possible to anticipate all the risks, uncertainties and the potential effects can be limited and reduced. These experts agree that this is a race against time, and the worst case scenario for the humankind within 30 years would be the discovery of a serious problem that could be detected at the beginning, as occurred with asbestos.

Researchers have made significant progress in understanding the effects of MNMs on some invertebrate and fish species, but the investigations of their interactions in a whole ecosystem or the microbial part of the food chain are still in their infancy. Furthermore, the surfaces of MNMs are engineered with a wide variety of designs that enhance their suitability for several industrial applications, but nature will modify these properties when they are released into the environment (Figure 1.9).[31]

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[26] Greßler S, Nentwich M. "Nano and Environment – Part II: Hazard potentials and risks. NanoTrust Dossier No. 027en - March 2012. Institute of Technology Assessment of the Austrian Academy of Sciences. 2012.

[27] Associate Dean of Research and Knowledge. Transfer and Chair Responsible Innovation. University of Exeter. The Business School.

[28] The nano revolution. Episode 3. Will nano save the planet? Directed by Mike Downie, produced by Takahiro Hamano. 2011. ARTE France DOCSIDE PRODUCTION-CBC NHK. Released in 2014.

[29] Department of Civil and Environmental Engineering. Duke University. Director of the Center for the Environmental Implications of NanoTechnology (CEINT).

[30] Assistant Professor of Environmental Science. Baylor University. Member of the Society of Environmental Toxicology and Chemistry (SETAC).

[31] Batley GE, Kirby JK, Mclaughlin MJ. **2013**. Fate and Risks of Nanomaterials in Aquatic and Terrestrial Environments. *Accounts Chem Res* 46:854-862.

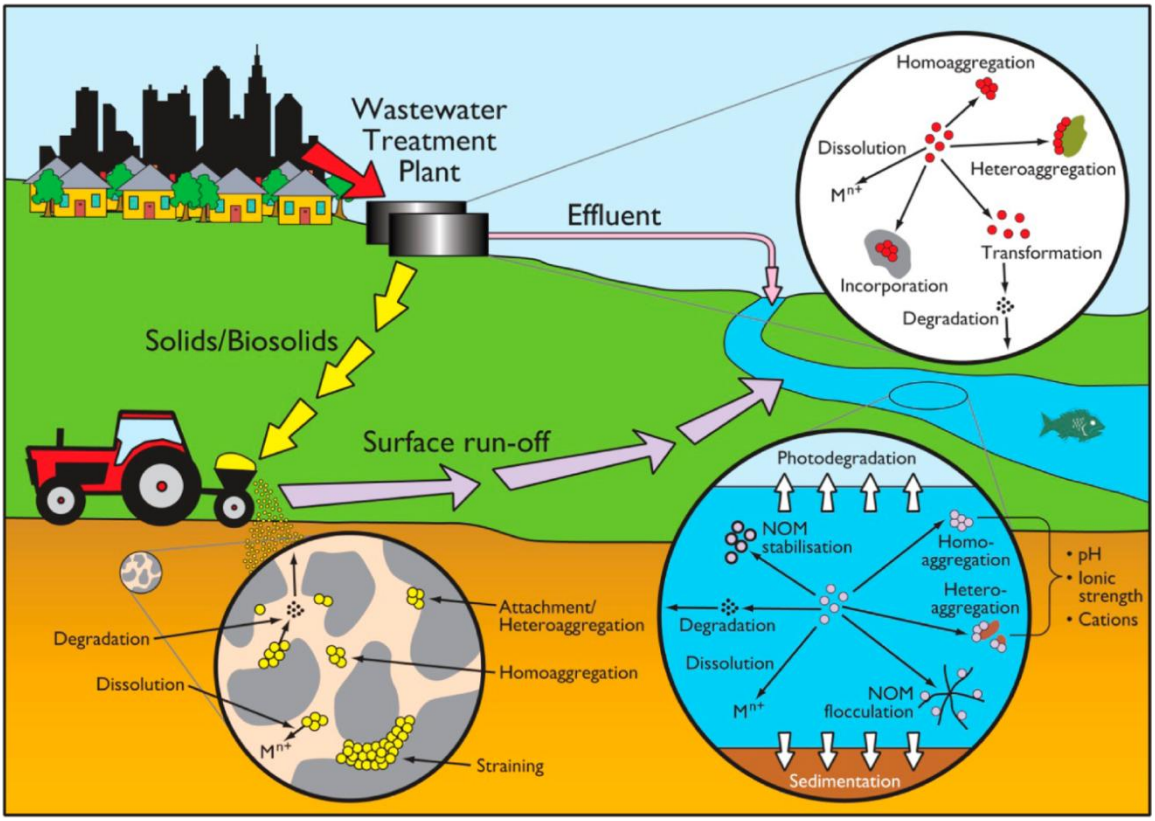

Figure 1.9 Pathways and transformations of nanomaterials in the environment.[31]
NOM = natural organic matter.

An efficient contribution of nanotechnology to the environmental and climate protection must go hand in hand with the reuse of nanomaterials and mitigation of their risks to avoid unintended consequences on the environmental integrity and human health. Several expert bodies agree that existing risk assessment methods for chemicals are to a large extent applicable to NMs. Nevertheless some aspects, including sample preparation and characterization, dosimetry, exposure data and models require further development of standardized and validated protocols.[23]

1.3. Approaching the nano-regulation

In the last few years, nanotoxicology has become an important topic of research with significant funding. Nonetheless, answers about risks of MNMs and their applications still cannot be properly addressed. The number of products on the market containing nanomaterials is growing steadily, and their development is rapidly evolving leading to subsequent increase in their applications and markets. The modifications of the properties of MNMs that enable promising applications might also result in new questions about how to predict, identify, measure, and monitor possibly risks for humans and the environment. Hence, answers are needed more quickly than can be obtained with conventional approaches, and the urgency of a nano-regulation has become evident.

At the international level various initiatives are aimed to fulfill the policy reforms needed for Environmental, Health and Safety (EHS) aspects of nanotechnology. In the last few years, the main efforts on the regulation of nano-safety worldwide have been focused on describing the state of the art of nanotoxicology, and only research prioritization tools or preliminary risk screening have been addressed.[32] This exploratory character is not applicable to support regulatory decision making, and specificity for nano-safety is not guaranteed by the existing regulations. For instance, the two pillars of the chemical safety regulation in the EU are the legal framework for placing chemicals on the market, and the specific provisions for health, consumer, occupational safety and environmental protection. Nevertheless, the testing schemes for MNMs are subject to discussion. Some reports have been provided based on OECD harmonized Test Guidelines, such as those under REACH requirements (Registration, Evaluation, Authorization and Restriction of Chemicals),[33] but it is uncertain whether they give appropriate information to perform toxicological assessments from a scientific point of view. Several approaches have fostered the capacity building and exchange of knowledge among the research community, such as the EU NanoSafety Cluster[34] or networking projects

[32] NANoREG Final Proposal: A common European approach to the regulatory testing of nanomaterials. European Union Seventh Framework Programme (FP7/2007-2013)-grant agreement n° 310584. Work programme topics NMP.2012.1.3-3 Regulatory testing of nanomaterials. 2012.
[33] http://ec.europa.eu/growth/sectors/chemicals/reach/nanomaterials/index_en.htm Last access: September 2015
[34] http://www.nanosafetycluster.eu/ Last access: September 2015

like NanoImpactNet[35] and QualityNano.[36] However, available data are scattered over many databases, hindering their full exploitation.

As a result of the CASG Nano meeting of November 2012,[37] the Netherlands sent a letter supported by Austria, Belgium, Croatia, the Czech Republic, Denmark, France, Italy, Luxembourg, Spain and Sweden, to urge the European Comission (EC) to take several measures for the nano-regulation.[38] They proposed to adapt the current legislation and establishing a register of MNMs and products containing them to raise awareness among consumers and workers as well as improve traceability. They also suggested adapting the REACH legislation regarding tonnage levels, registration deadlines and information requirements for MNMs.

The analyses conducted by REACH, the US-National Nanotechnology Initiative and the OECD WPNM (OECD's Working Party on Manufactured Nanomaterials) similarly has concluded that reference materials and standardized methods for assessment of hazards and risks are necessary before guidance can be adapted to MNMs.[32] The US Research Strategy for EHS Aspects of Engineered Nanomaterials[39] distinguished four research categories of higher priority: 1) identification, characterization, and quantification of the origins of nanomaterial release, 2) processes that affect potential hazards and exposure, 3) nanomaterial interactions in complex systems ranging from subcellular systems to ecosystems and 4) adaptive research and knowledge infrastructure for accelerating research progress and providing rapid feedback to advance research. In October 2012, the Second Regulatory Review on Nanomaterials[40] was published by the EC (Figure 1.10). It concluded that the risk assessment of MNMs should be performed on a case-by-case basis. The challenges were related primarily to establishing validated methods and instrumentation for detection, exposure, characterization, and completing information on hazards. The Commission proposed REACH as the best possible framework for the risk

[35] http://www.nanoimpactnet.eu/ Last access: September 2015
[36] http://www.qualitynano.eu/ Last access: September 2015
[37] http://ec.europa.eu/environment/chemicals/nanotech/reach-clp/caracal_en.htm
Last access: September 2015
[38] Bleeker EAJ, Theodori D, Wijnhoven SWP. Exploring Building Blocks for Amending EU Regulation of Nanomaterials, National Institute for Public Health and the Environment, Netherlands. 2013.
[39] A Research Strategy for Environmental, Health, and Safety Aspects of Engineered Nanomaterials. National Research Council of the National Academies. The National Academies Press, Washington D.C., 2012.
[40] Second Regulatory Review on Nanomaterials. Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee Brussels. 3 October 2012.

management of MNMs, envisaged modifications in some of the REACH Annexes, and encouraged ECHA to further develop guidance for registrations after 2013.

Figure 1.10 Web of the European policy issues on nanotechnology.[41]

In the EU´s Seventh Framework Programme (European Union's Research and Innovation funding programme for 2007-2013), the European Commission massively increased funding for research projects that dealt with the risk- and safety-relevant aspects of nanotechnologies. Priority was also given to projects that featured a higher level of networking both in their thematic scope and among participating institutions. An additional focus was placed on the funding of projects designed to promote information processing and the exchange of knowledge.[42]

The EU-FP7 NANoREG project[32] has provided an essential framework for the present study throughout IK4-Tekniker, one of its 70 participating partners. This project is

[41] http://ec.europa.eu/research/industrial_technologies/the-policy_en.html
Last access: September 2015

[42] Fries R, Gazsó A. Research projects on EHS aspects of nanotechnology in the 7th Framework Program of the EU. NanoTrust Dossier No. 030en - May 2012. Institute of Technology Assessment of the Austrian Academy of Sciences. 2012.

aimed to give an answer to regulators and legislators on EHS aspects of MNMs by linking regulatory questions and needs to a scientific evaluation of data and test methods. The most important regulatory questions identified by consultation with regulators were related to measurements, characterization, transformation, dose metrics, grouping, persistence and long-term effects, kinetics, mode of action, hazard, exposure, risk assessment and management and health surveillance.[43]

NANoREG has developed building blocks for the regulatory testing of MNMs.[44] It has been aimed at "testing the test" to identify appropriate ways to assess the EHS effects of nanomaterials. The Project has used a cross-cutting suite of test materials and the same characterization methods in order to harmonize the test procedures and generate reproducible as well as comparable information. A centralized data sharing platform has collected the results obtained on a specific format, which will be made available to the legislation authorities for the regulatory assessment (Figure 1.11).

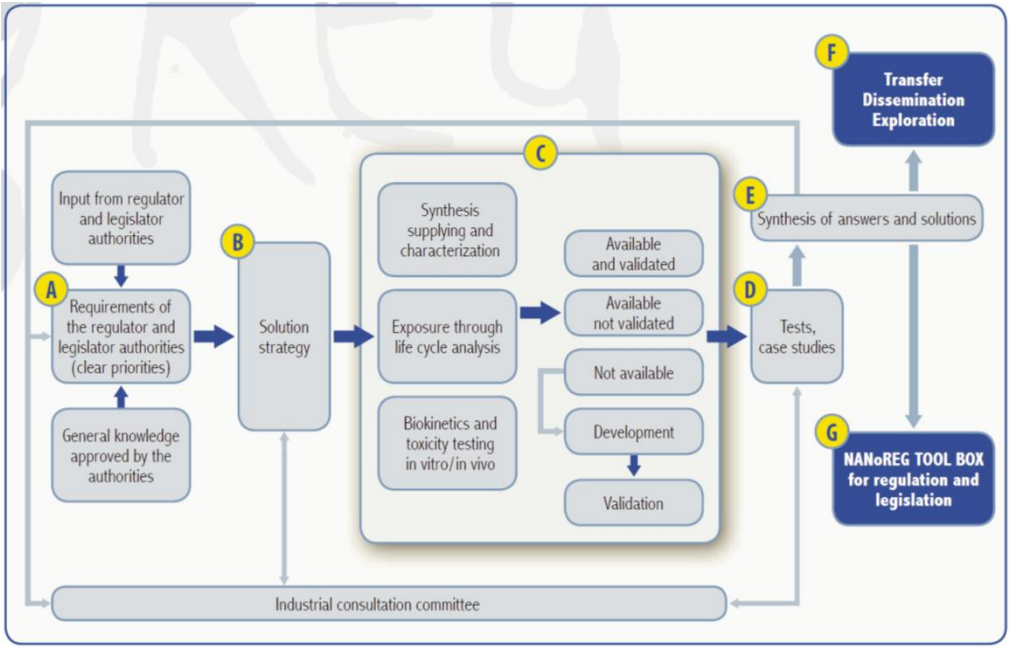

Figure 1.11 NANoREG Workflow.[45]

[43] NANoREG Newsletter 3-March 2015. http://nanoreg.eu/index.php/news-events/newsletter.html Last access: September 2015

[44] NANoREG Newsletter 2-August 2014. http://nanoreg.eu/index.php/news-events/newsletter.html Last access: September 2015

[45] NANoREG Factsheet 10-June 2013. http://nanoreg.eu/index.php/media-and-downloads.html Last access: September 2015

The establishment of a channel of communication with the regulatory authorities and industry has been a key issue for NANoREG. A specific work package is responsible for liaising with international organisations such as OECD, CEN (European Committee for Standardization), ISO, as well as handling global relationships with US, Canada, Asia, and Australia. National Coordinators were installed as direct links to the different authorities (human health, environment, workers safety, pharma, the R&D community, industry and the public) in each partner country. NANoREG has addressed the international stakeholders, coordinating and exchanging information on scientific results, regulation and legislation approaches as well as standardization. The Project has additionally created an "Industry Consultation Committee", where industry can influence the outcome of the project before regulation will take place, steer national and OECD policies, and achieve a high profile in public awareness by a responsible approach to these issues. NANoREG results are being transferred to the regulation and legislation authorities with workshops on a national and international scale.

1.4. Ecotoxicity and environmental fate of manufactured nanomaterials

As highlighted in Chapter 1.2, natural resources and biodiversity constitute fundamental assets for the survival of all kind of live in the Earth. Ecotoxicology is a relatively new science concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context.[46] Until 2008, a substantial number of studies had investigated the toxicity of MNMs in bacteria, mammals and mammalian cell lines, but the scientific discipline of nanoecotoxicology was still in its infancy.[47] The evolution of the number of publications addressing the potential risks of MNMs for the environment is presented in Figure 1.12. Although this analysis has attracted increasing interest, significant scientific uncertainties and contradictory results related to their toxicity and exposure characteristics still remain.[48,49]

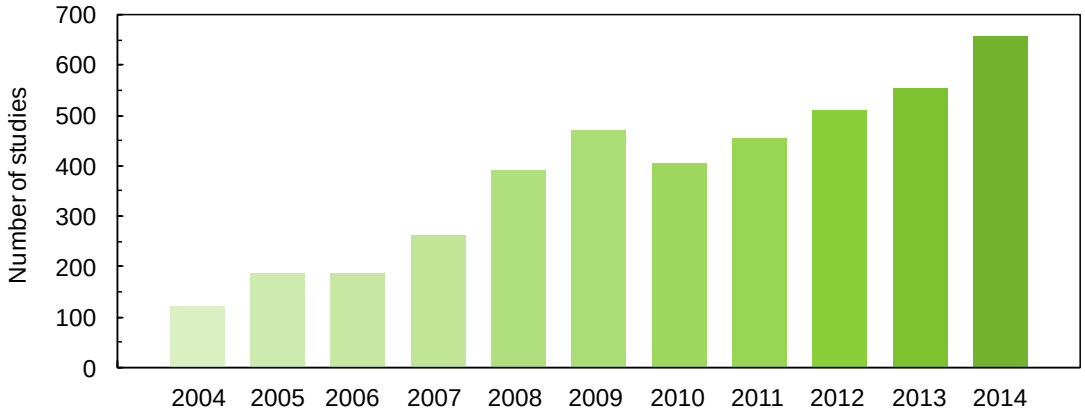

Figure 1.12 Evolution over the past decade of the number of studies identified on the potential risks of MNMs for the environment. The analysis was conducted using Web of Science, and literature searches were performed using the term nano* in combination with ecol*, ecosystem*, environment* and ecotox* in the field "Title".

The available literature concerning the models of potential release of MNMs into the environment has shown that they are expected to be found in the different

[46] Kahru, A., Dubourguier, H-C. **2010**. From ecotoxicology to nanoecotoxicology. *Toxicology* 269:105-119.
[47] Baun A, Hartmann NB, Grieger K, Kusk KO. **2008**. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotoxicology* 17:387-395.
[48] Beaudrie CEH, Satterfield T, Kandlikar M, Harthorn BH. **2013**. Expert views on regulatory preparedness for managing the risks of nanotechnologies. *Plos One* 8:e80250.
[49] Djurisic AB, Leung YH, Ng AMC, Xu XY, Lee PKH, Degger N, Wu RSS. **2015**. Toxicity of metal oxide nanoparticles: mechanisms, characterization, and avoiding experimental artefacts. *Small* 11:26-44.

environmental compartments (i.e., soil, water, air and landfills).[24,25,50] Thus, further investigation is required to elucidate their potential effects on the health of ecosystems. The main problems that should be solved in the field of nanoecotoxicology are related to the choice and characterization of MNMs to use in biological experiments, the organisms tested and endpoints measured, and the routes of exposure and interactions with organisms in different environments.[46]

Concerning the prioritization of testing MNMs, several lists have been identified by various groups, namely the NIST (US National Institute of Standards and Technology) and the OECD, as being important for risk assessment. Twenty-five individual nanomaterials are under consideration and have been prioritized for development as candidate reference materials worldwide.[51] These MNMs can help researchers to develop harmonized protocols to elucidate their toxicity mechanisms and verify method performance and laboratory proficiency, improving consistency in interpreting exposure and toxicity data. The OECD list of priority nanomaterials included fullerenes (C60), single-walled carbon nanotubes (SWCNTs), multiwalled carbon nanotubes (MWCNTs), dendrimers, nanoclays, and silver, gold, iron, titanium dioxide, aluminium oxide, cerium oxide, zinc oxide and silicon dioxide nanoparticles.[52] The evaluation of the literature performed in the framework of the NANoREG project mapped 29 main applications for the core set of MNMs, considering factors such as volume of European production and existing exposure data.[53] The map gives an overview of all relevant tasks along the life cycle of a nanomaterial, i.e., synthesis, functionalization, formulation, use and end of life. For all processes, descriptors of use and determinants of exposure were collected to rate and rank the exposure scenarios (Figure 1.13).

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[50] Gottschalk F, Sun T, Nowack B. **2013**. Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies. *Environ Pollut* 181:287-300.

[51] Stefaniak AB, Hackley VA, Roebben G, Ehara K, Hankin S, Postek MT, et al. **2013**. Nanoscale reference materials for environmental, health and safety measurements: needs, gaps and opportunities. *Nanotoxicology* 7:1325-1337.

[52] List of Manufactured Nanomaterials and List of Endpoints for Phase One of the Sponsorship Programme for the Testing of Manufactured Nanomaterials: Revision. OECD Environment, Health and Safety Publications Series on the Safety of Manufactured Nanomaterials No. 27. ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT. Paris, 2010.

[53] NANoREG Factsheet Deliverable 3.1-Critical exposure scenarios. 20-May 2015.
http://nanoreg.eu/index.php/media-and-downloads.html
Last access: September 2015

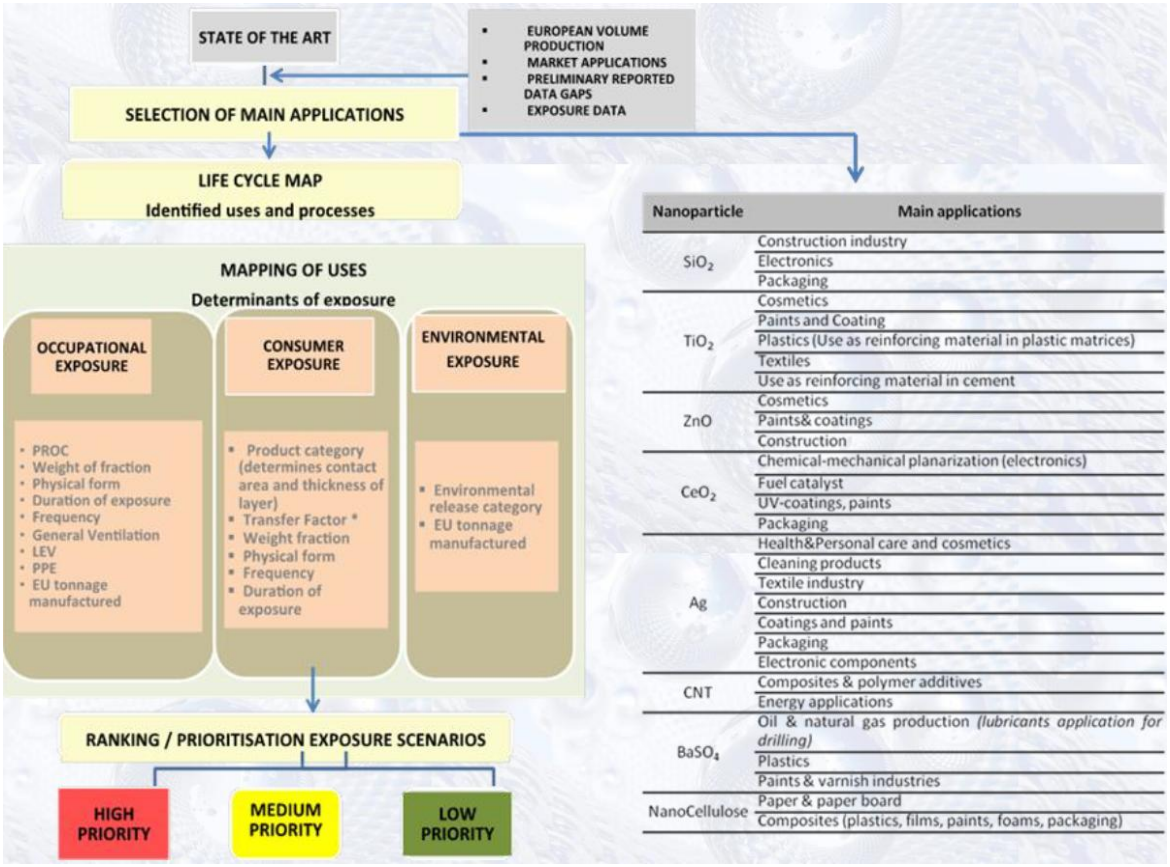

Figure 1.13 Approach for ranking/prioritizing exposure scenarios in the NANoREG project.[53]

The choice of organisms and endpoints measured constitute another major issue faced by nanoecotoxicology. While risk assessment for human health concerns one species, environmental risk assessment should ideally consider millions of species, but it consists of a gross simplification of an ecosystem. Indeed, a battery of biotests with different sensitivity profiles involving organisms of different trophic levels is often recommended and used. The tests for the regulatory decision-making are selected in terms of cost, ecological relevance, reproducibility and sensitivity (Figure 1.14). For example, the recommended model organisms for classification and labeling of ecotoxicological hazard of chemicals are fish (OECD Guideline 203),[54] *Daphnia* (OECD Guidelines 202, 211),[55,56] and algae (OECD Guideline 201).[57] In order to

[54] Fish, Acute Toxicity Test. OECD Guideline 203. Organization for Economic Cooperation and Development (OECD). Paris, France. Adopted 17 July 1992.
[55] Daphnia sp. Acute Immobilisation Test. OECD Guideline 202. Organization for Economic Cooperation and Development (OECD). Paris, France. Adopted 13 April 2004.
[56] Daphnia magna Reproduction Test. OECD Guideline 211. Organization for Economic Cooperation and Development (OECD). Paris, France. Adopted 2 October 2012.
[57] Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD Guideline 201. Organization for Economic Cooperation and Development (OECD). Paris, France. Adopted 23 March 2006, corrected 28 July 2011.

avoid unnecessary use of animals as well as reduce testing costs, it is widely agreed that all information on a substance relevant to its potential ecotoxicity should be evaluated prior to considering testing in fish, the main vertebrate test organism.[46] The effects of MNMs should also be determined in different stages of the food chain, and to date, significant efforts have been made to assess their ecotoxicity in a variety of micro and macro organisms. Nonetheless, specific standardized protocols or certified reference materials for the MNMs testing do not exist, thus making difficult an analysis of the data obtained. In addition, different parameters have been assessed as test endpoints. Some researchers have reported LC50 (lethal concentration 50%) while others MIC (minimum inhibitory concentration).[58]

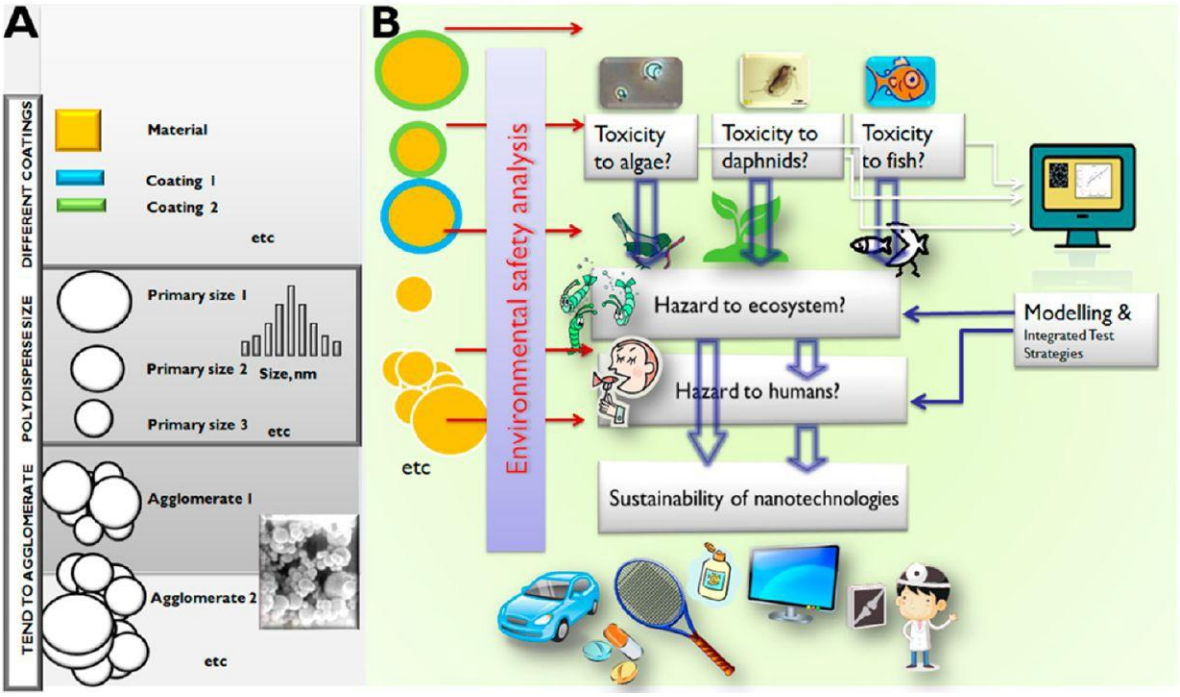

Figure 1.14 The challenges of understanding the environmental risks of MNMs. (A) One type of NM may yield tens to hundreds of (nano) particulate entities with different physicochemical properties. (B) Obtaining toxicity (dose-effect) data for a chemical/substance for a set of environmentally relevant key organisms is a crucial step for risk assessment.[59]

[58] Peralta-Videa JR, Zhao L, Lopez-Moreno ML, de la Rosa G, Hong J, Gardea-Torresdey JL. **2011**. Nanomaterials and the environment: a review for the biennium 2008-2010. *J Hazard Mater* 186:1-15.

[59] Kahru A, Ivask A. **2013**. Mapping the dawn of nanoecotoxicological research. *Accounts Chem Res* 46:823-833.

Regarding the routes of exposure to MNMs in ecosystems, different pathways and interactions have been identified by Navarro et al.[60] (Figure 1.15). The effects of MNMs on photosynthetic organisms may reduce the fixation of CO_2 (1); NMs adsorbed (2) or deposited (3) on photosynthetically active surfaces might reduce light availability or gas exchange (4) and thus photosynthesis; NMs present in the atmosphere might increase the nuclei available for raindrop formation (5), thus altering precipitation; NMs' impacts on bacteria, fungi, and other edafic fauna (6) might affect soil respiration (7), and other soil-texture-related processes such as transport of liquids (8) or gases (9), also modifying symbiotic relationships (10). Together, this might lead to impairments in three key services provided by ecosystems, i.e., nutrient cycling (11), water depuration (12), and biomass production (13).

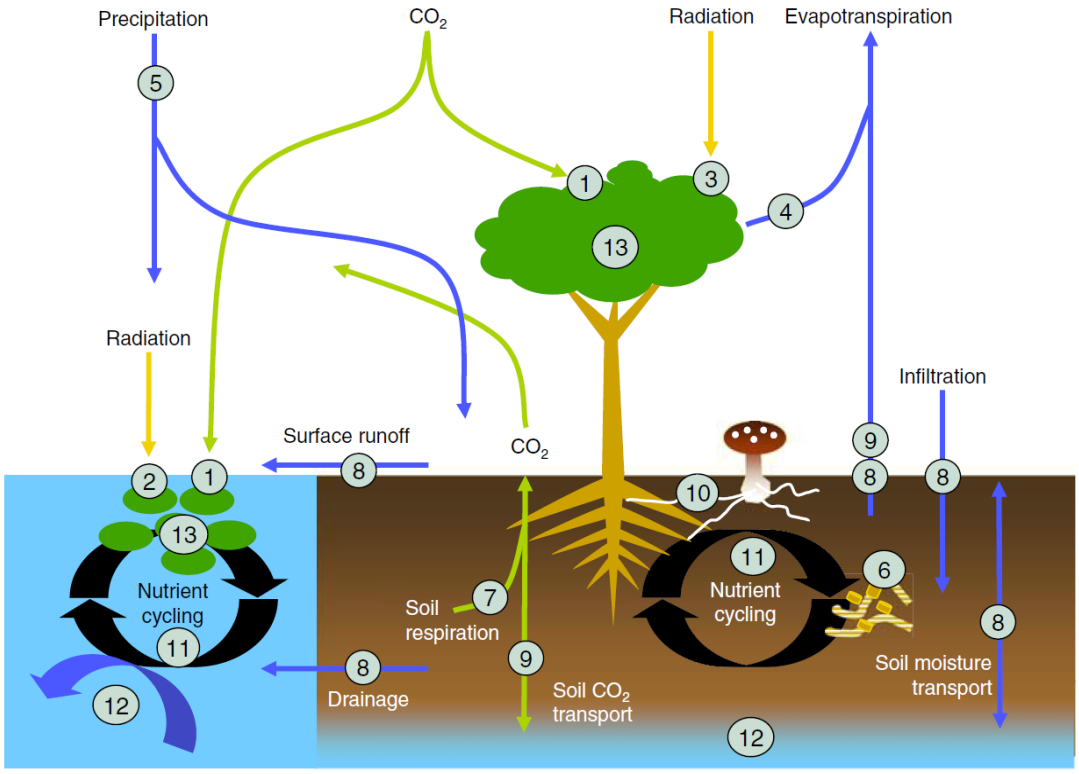

Figure 1.15 Simplified schemes of the pathways of MNMs into the environment and some terrestrial and aquatic ecosystem processes that might be altered.[60]

The ecological relevance of the effects of MNMs in aquatic and terrestrial ecosystems is also influenced by many processes that may not be relevant to

[60] Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao A-J, Quigg A, Santschi PH, Sigg L. **2008**. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology* 17:372-386.

traditional contaminants, such as instability, agglomeration, dissolution, deposition, and attachment. These are all determined by the ambient environmental characteristics and the size and surface properties of the nanomaterials (Figure 1.16).[61] Taking into consideration that some MNMs dissolve over time, the exposure can consist of both the suspended nanomaterials and the dissolved ions. In addition, transformation processes such as agglomeration might result in an unexpected behavior. For instance, agglomerates interact with the environment in different ways and rates compared with individual NPs or dissolved ions. MNMs are also susceptible to transformation processes such as acquiring coatings that alter their original properties and environmental effects (e.g., oxidation, sulfidation). Given the uncertainties in the emissions of MNMs into the environment, interactions with natural colloids and natural organic matter (NOM), and the effect of environmental properties, definite conclusions about their fate and transport patterns are limited.

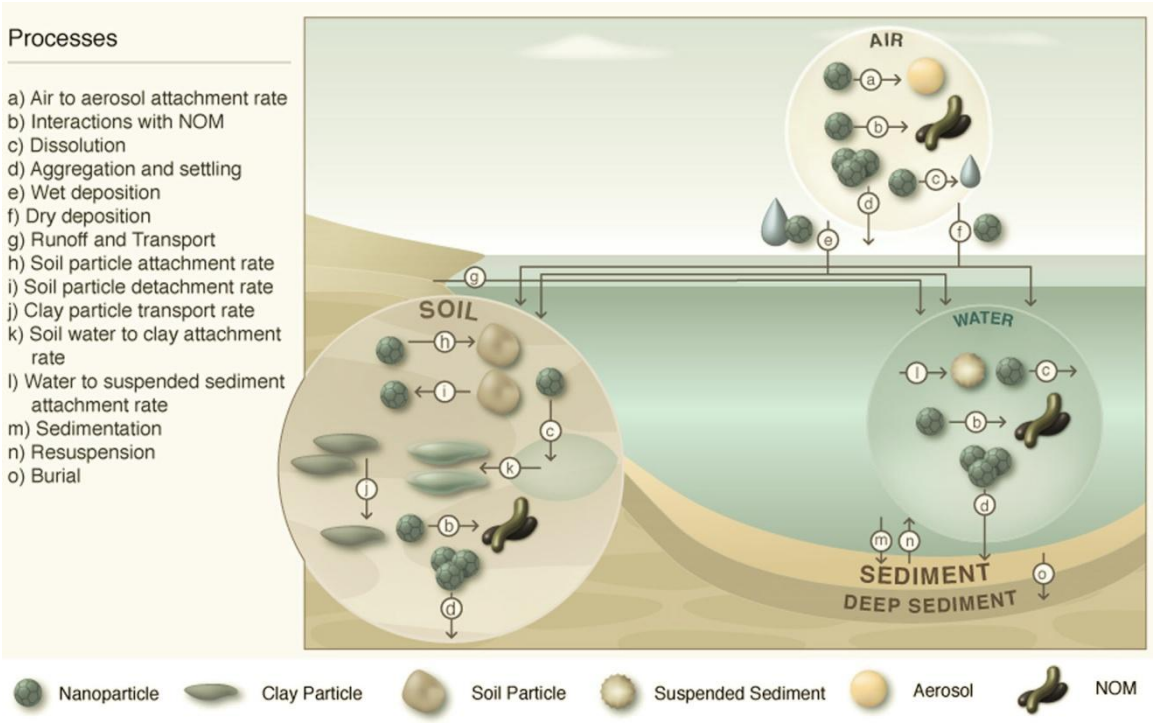

Figure 1.16 Conceptual model of key MNMs fate processes: Transport of nanoparticles between environmental compartments and their interactions with other constituents in the environment as well as with themselves.[61]

Among the multiple pathways of MNMs into the ecosystems, aquatic organisms constitute one of the most important for their entrance and transfer throughout the

[61] Garner KL, Keller AA. **2014**. Emerging patterns for engineered nanomaterials in the environment: a review of fate and toxicity studies. *J Nanopart Res* 16:2503.

food web. Surface waters receive pollutants from atmospheric deposition, leaching from soil and through direct inputs, such as run-off and wastewater discharges from domestic and industrial sources. Surface water bodies can also import water from groundwater reservoirs, transporting with it MNMs. Furthermore, the aquatic environment has been targeted for some nano-scale environmental remediation techniques.[47, 62]

The behavior of MNMs in natural aquatic environments is dependent on particle-specific properties (e.g., size, shape, chemical composition, surface charge, and coating), particle state (free or matrix incorporated), the surrounding solution chemistry (e.g., pH, ionic strength, ionic composition, natural organic matter content), and hydrodynamic conditions.[63,64] Such factors are important in determining whether particles aggregate or agglomerate with other particles, dissolve or sediment onto various environmental surfaces.[31] Recognizing these interactions under different conditions is essential in predicting their fate in aquatic ecosystems and thus likelihood of exposure.

Despite some methods for detecting and characterizing MNMs in natural waters have been developed, such as field flow fractionation coupled and inductively coupled plasma mass spectrometry, the information on levels in aquatic environments is scarce.[62] There are still major knowledge gaps on production, application and release of nanomaterials that affect the modeled values. Nonetheless, an agreement on the order of magnitude of the environmental concentrations can be reached. Gottschalk et al.[50] demonstrated that a certain understanding of the expected MNMs concentrations in surface waters is possible through the comparison of modeled concentrations and real measurements. They validated the predicted concentrations only to a minor extent, but provided useful data for the environmental risk assessment of MNMs (Figure 1.17).

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[62] Scown TM, van Aerle R, Tyler CR. **2010**. Review: Do engineered nanoparticles pose a significant threat to the aquatic environment? *Crit Rev Toxicol* 40:653-670.

[63] Petosa AR, Jaisi DP, Quevedo IR, Elimelech M, Tufenkji N. **2010**. Aggregation and deposition of engineered nanomaterials in aquatic environments: role of physicochemical interactions. *Environ Sci Technol* 44:6532-6549.

[64] Keller AA, Wang H, Zhou D, Lenihan HS, Cherr G, Cardinale BJ, Miller R, Ji Z. **2010**. Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices. *Environ Sci Technol* 44:1962-1967.

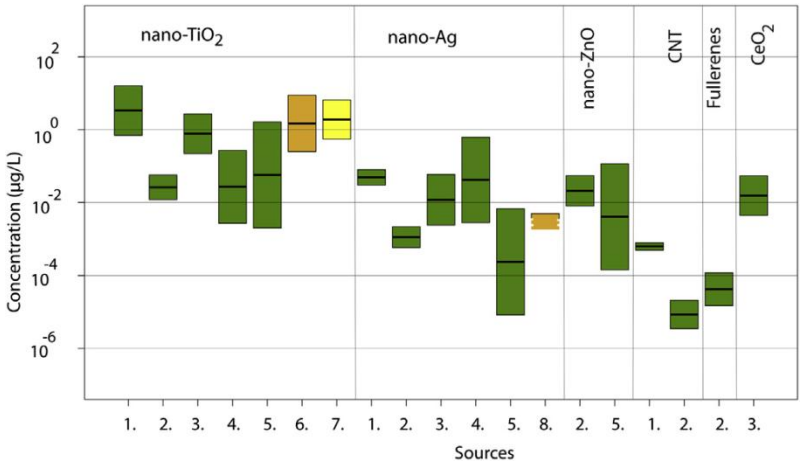

Figure 1.17 Modeled and analytical concentrations of MNMs in surface waters. The green boxes show the range (and the arithmetic mean on the logarithmic scale) of modeled results, yellow boxes measured concentrations and the orange boxes combine measurements and modeling.[50] CNT = carbon nanotube.

As expected, Figure 1.17 shows that the MNMs included in the various priority lists for risk assessment described previously are also susceptible to be released into aquatic systems. Garner et al.[61] classified some of them as the most persistent in freshwater systems, although the type of water was a key factor in their environmental fate. Most MNMs are expected to aggregate to some extent after release (Figure 1.18), and their behavior might be very different from that of primary MNM. The degree of agglomeration depends on the characteristics of the aquatic system (freshwater and stormwater provided increased stability) but also on the physicochemical properties and MNM concentrations in colloidal suspensions.

Residence Time	Stormwater	Freshwater	Groundwater	Seawater
Months	Ag* Au* CeO_2 C_{60} FeO/Fe_2O_3 MWCNTs SiO_2 TiO_2 ZnO	Ag* Au* CeO_2 C_{60} FeO/Fe_2O_3 MWCNTs SiO_2 TiO_2 ZnO	C_{60} MWCNTs SiO_2	SiO_2
Weeks	nZVI* SWCNTs	NiO nZVI* SWCNTs	Au* FeOOH Latex SWCNTs	CuO FeO/Fe_2O_3*
Days		Al_2O_3	Ag* CeO_2 CuO NiO TiO_2	Ag* C_{60} FeOOH SWCNTs
Hours			FeO/Fe_2O_3 nZVI* ZnO	Au* CeO_2 MWCNTs nZVI* TiO_2 ZnO

Figure 1.18 Predicted agglomeration time frames of MNMs in different water types.[61] * = coated nanomaterial; nZVI = nano zero valent iron.

The initial particle concentration affects the collision frequency, and the macromolecular components, such as NOM, affect the attachment efficiency. In general, they are the two main parameters influencing deposition of MNMs to the sediment compartment, following the agglomeration process.[65] Faster sedimentation results in a shorter residence time of MNMs in water. In general, sedimentation and agglomeration are faster in seawater than in the other water types. In freshwater and stormwater, particles are likely to remain suspended for extended lengths of time (Figure 1.19). Hence, the exposure to MNMs is expected to be higher for freshwater aquatic and benthic marine species.[61,66]

Residence Time	Stormwater	Freshwater	Groundwater	Marine
Months	Au* CeO_2 C_{60} MWCNTs SiO_2 SWCNTs TiO_2	Au * C_{60} MWCNTs SiO_2 SWCNTs TiO_2	C_{60} FeOOH SiO_2	SiO_2
Weeks	Ag* ZnO	Ag* CeO_2 CuO NiO ZnO	Au* CeO_2 NiO ZnO	Au
Days	nZVI*	FeO/Fe_2O_3 nZVI*	nZVI* TiO_2	Ag* C_{60} FeOOH nZVI* ZnO
Hours			FeO/Fe_2O_3	CeO_2 MWCNTs TiO_2

Figure 1.19 Predicted sedimentation time frames of MNMs in different water types, as measured by the residence time in the water column.[61] * = coated nanomaterial; nZVI = nano zero valent iron.

Dissolution of metallic MNMs results in the release of metal ions and their disappearance. MNMs that dissolve require close monitoring because the ionic form of a metal may cause more significant adverse effects than the nanomaterial.[67] With the exception of ZnO in seawater and freshwater, NP dissolution is generally slow and does not vary significantly by water type (Figure 1.20). This means that many MNMs will remain in nanoparticle form or within aggregates/agglomerates for significant periods of time. This will lead to high exposure of freshwater aquatic

[65] Quik JTK, Stuart MC, Wouterse M, Peijnenburg W, Hendriks AJ, van de Meent D. **2012**. Natural colloids are the dominant factor in the sedimentation of nanoparticles. *Environ Toxicol Chem* 31:1019-1022.

[66] Erhayem M, Sohn M. **2014**. Effect of humic acid source on humic acid adsorption onto titanium dioxide nanoparticles. *Sci Total Environ* 470-471:92-98.

[67] Mallevre F, Fernandes TF, Aspray TJ. **2014**. Silver, zinc oxide and titanium dioxide nanoparticle ecotoxicity to bioluminescent Pseudomonas putida in laboratory medium and artificial wastewater. *Environ Pollut* 195:218-225.

species or benthic marine species (depending on their stability) to particulate MNMs rather than dissolved MNMs.

Residence Time	Stormwater	Freshwater	Groundwater	Marine
Months	Au* FeO/Fe_2O_3 NiO TiO_2	Au* CeO_2 FeO/Fe_2O_3 TiO_2	Au* CeO_2* CuO NiO TiO_2	Au* CeO_2 CuO TiO_2
Weeks	CuO ZnO	Ag* Al_2O_3 CuO NiO PbS	Ag* ZnO nZVI*	Ag* Al_2O_3 NiO
Days		ZnO		
Hours				ZnO

Figure 1.20 Predicted dissolution time frames of MNMs in different water types.[61] * = coated nanomaterial; nZVI = nano zero valent iron.

Agglomeration and deposition behavior will dictate MNMs transport potential and thus their environmental fate, bioavailability, and ecotoxicological impacts. Most of them are largely stable in freshwater, which will result in greater likelihood of exposure and hence, increased adverse effects (Figure 1.21). MNMs might exhibit low mobility in marine systems because of the higher rates of agglomeration and sedimentation observed with respect to freshwater.[61]

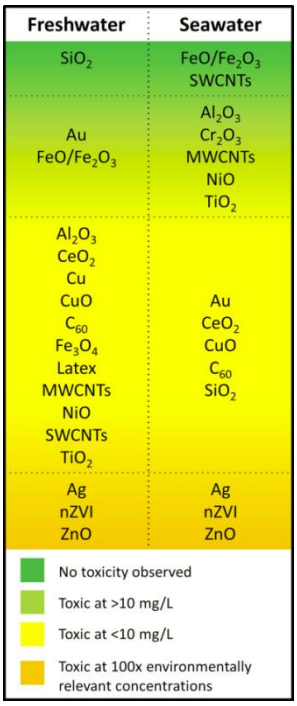

Figure 1.21 Toxicity of MNMs in freshwater and marine systems corresponding to 61 MNMs ecotoxicity studies.[61] nZVI = nano zero valent iron.

In 2014, the OECD published a meeting report[68] discussing the applicability of existing OECD Test Guidelines (TGs) to the fate and ecotoxicity of MNMs, in order to identify whether there was a need to amend current TGs or developing new ones. It was concluded that a better understanding of the complex interactions between MNMs and environmental compartments should be achieved in order to give specific recommendations for their ecotoxicological assessment. Specifically, the advice regarding aquatic ecotoxicity covered three main issues: the preparation of the stock/stem suspensions, the preparation of the exposure solution, and the test requirements (Table 1.3).

Table 1.3 Recommendations on aquatic ecotoxicology assessment of MNMs (OECD).[68]

	Recommendations
Stock suspension	1. Using the same stock suspensions for both aquatic ecotoxicology and environmental fate testing (comparability of results amongst tests and minimization of test artefacts).
	2. Preparation method that avoids unintended MNMs damage, repeatable and achievable with standard equipment.
	3. Stock suspension as stable and monodisperse as possible. Stable = retaining a certain size range for a certain time (ideally the duration of a test).
	4. Undertake pilot runs to determine applied energy needs, stability with or without stabilizers, such as NOM. The type of NOM must be specified given that different types of NOM may have different effects.
Exposure solution	5. Consider that the properties of MNMs will change upon the addition of more complex exposure media (e.g. presence of proteins, sugars, salts).
	6. Considering alternative dose metrics for varying exposure (nominal concentration as worst case; geometric mean etc.).
Test requirements	7. Measurement of particle number, mass, surface area or ion release may be necessary.
	8. Results from high exposure concentrations should be considered in the context of more realistic (lower) environmental exposures.
	9. Running pilot tests to determine material loss.
	10. Testing MNMs which reflect most likely transformations after their introduction into environment (species resulting from ligand binding).

[68] Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report OECD Environment, Health and Safety Publications. Series on the Safety of Manufactured Nanomaterials No. 40. ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT. Paris, 2014.

All chemical products, including MNMs, produced in EU by more than one metric ton per year, need to be ecotoxicologically characterized by 2018.[59] MNMs are inherently polydisperse; that is, vary in size and often in coating. Thus, a single nanomaterial may present a huge number of combinations of entities with different physicochemical properties, leading to differences in their environmental behavior. Given the substantial diversity within each group of MNMs and the complexity of nanosystems, the assessment of the overall hazard of a single MNM type must consider a large number of property combinations. Currently, it is widely accepted among scientists and regulators that it can only be addressed on a case-by-case basis. However, it would be a time and resource intensive task considering the large number of existing and emerging nanoformulations.[69] Integrated test strategies or computational models to avoid testing all the possible nano-particulate entities are currently receiving increasing attention, and specific Intelligent Testing Strategies (ITS) have already been approached.[70] Computational methods based on Quantitative structure-activity relationship (QSAR) are already relatively common theoretical models in regulatory risk assessment of chemicals. The QSAR paradigm is based on the assumption that the variance in biological activity of the compounds is determined by the variance in their molecular structure.[71] This means that if some molecular parameters for a group of compounds have been measured (or calculated) and toxicological data are available only for a part of this group, the unknown data can be interpolated from the molecular descriptors and a suitable mathematical model. Computational methods might also be a powerful alternative in the prediction of the interactions of MNMs with living systems and the environment, and preliminary works exist on QNAR (Quantitative nanostructure-activity relationship) models (Figure 1.22).[72-74]

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[69] Oomen AG, Bos PMJ, Fernandes TF, Hund-Rinke K, Boraschi D, Byrne HJ, et al. **2014**. Concern-driven integrated approaches to nanomaterial testing and assessment - report of the NanoSafety Cluster Working Group 10. *Nanotoxicology* 8:334-348.

[70] Stone V, Pozzi-Mucelli S, Tran L, Aschberger K, Sabella S, Vogel U, et al. **2014**. ITS-NANO - Prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. *Part Fibre Toxicol* 11:9.

[71] Puzyn T, Leszczynska D, Leszczynski J. **2009**. Toward the development of "nano-QSARs": advances and challenges. *Small* 5:2494-2509.

[72] Chau YT, Yap CW. **2012**. Quantitative Nanostructure-Activity Relationship modelling of nanoparticles. *RSC Adv* 2:8489.

[73] Lynch I, Weiss C, Valsami-Jones E. **2014**. A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a basis for safer-by-design NMs. *Nano Today* 9:266-270.

[74] Westerhoff P, Nowack B. **2013**. Searching for global descriptors of engineered nanomaterial fate and transport in the environment. *Accounts Chem Res* 46:844-853.

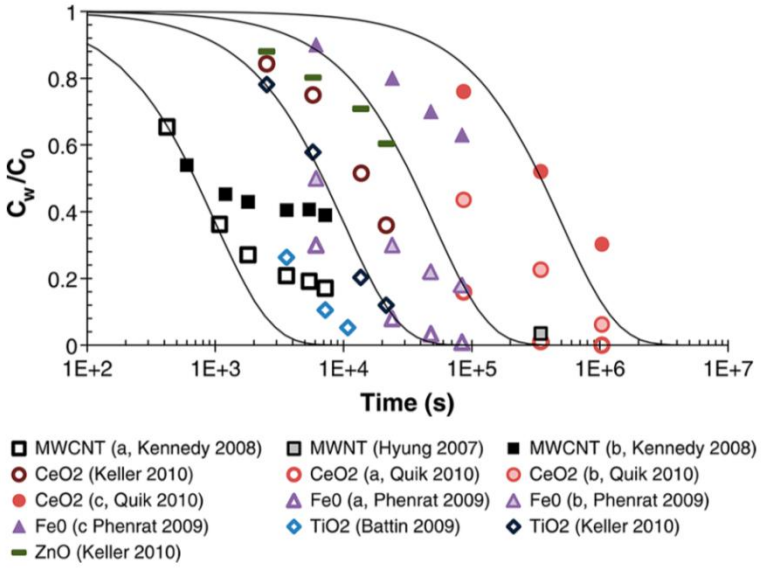


Figure 1.22 Fraction of MNMs remaining in suspension over time (filled symbols, with NOM; open symbols, without NOM). The lines represent a first-order removal model.[74]

However, these models are of limited use even for conventional chemicals, mostly due to the lack of good-quality experimental toxicity data. Furthermore, the complexity of the structural descriptors of MNMs leads to additional difficulty of QNARs compared to QSAR models.[59] In order to establish reliable correlations between the ecotoxicological profiles and the physicochemical properties of MNMs, a previous study is necessary at least for a core set of them at various trophic levels.

The situation described above shows the urgent need for the definition of test methods to overcome the limitations of the toxicological assessment of MNMs in aquatic ecosystems.

Chapter 2. Hypothesis

Nanotechnology involves the manipulation of the physicochemical properties of matter at the nanometre scale, which holds promise for innovative solutions to various scientific disciplines, such as physics, chemistry, information technology, medicine or biology. However, the novel features of manufactured nanomaterias (MNMs) lead to questions about their physical, health and environmental risks. These uncertainties might result in polarized public debate and business unwillingness to invest further in nanotechnology. One of the most important pathways for the entrance of MNMs and their transfer throughout the food web is represented by aquatic organisms, but the lack of standardized assessment protocols is leading to contradictory toxicity results in natural waters. Although expert organizations agree that existing methods are to a large extent applicable to MNMs, certain aspects (sample preparation and characterization, dosimetry, exposure data and model organisms) require further validation and regulation.

The present study was intended to define specific test methods to overcome the limitations of the toxicological assessment of MNMs in aquatic ecosystems. Organisms of different trophic levels, selected in terms of cost, ecological relevance, reproducibility and sensitivity, were planned to be tested in the ecotoxicological analysis (seawater bacterium *V. fischeri* and the freshwater crustacean *D. magna* and microalgae *P. subcapitata*). Multiwalled carbon nanotubes (MWCNTs) and TiO_2 and CeO_2 nanoparticles were selected as the MNMs subject of study. They are currently included in the prioritization lists of relevant reference materials worldwide, defined considering production volume, main market applications and persistence in the environmental compartments.

The standardization approach of the current work was also scheduled to be fulfilled by considering two relevant aspects, which were expected to improve the colloidal stability of MNMs and the reproducibility of the ecotoxicity test results. Firstly, the optimization of the energy delivered to the MNMs during the preparation of the aqueous dispersions, and secondly, the addition of a reference natural organic matter in the aqueous media of the organisms. The presence of this substance was also intended to allow conducting the exposures in environmentally realistic conditions.

Chapter 3. Objectives

The present thesis is based on the necessity of regulating and validating test methods to overcome the current limitations of the toxicological assessment of MNMs in aquatic ecosystems.

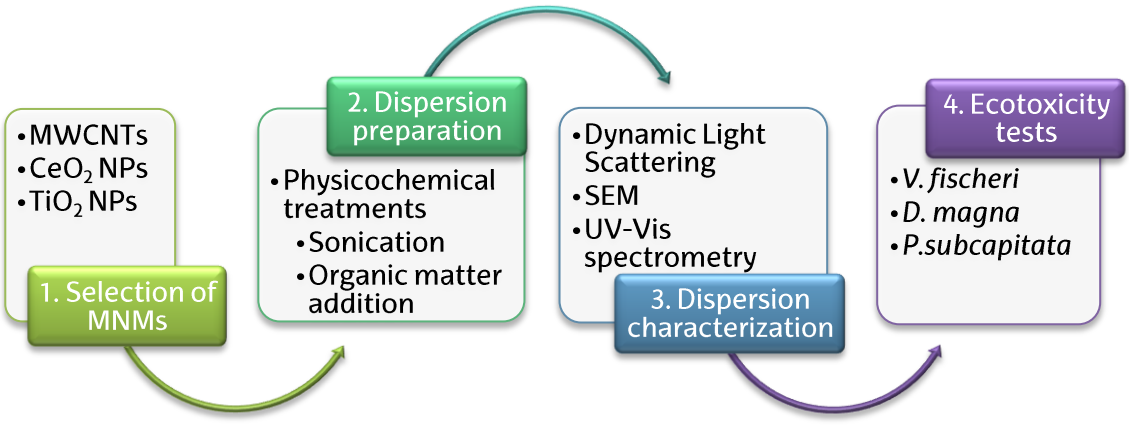
Multiwalled carbon nanotubes (MWCNTs) and TiO_2 and CeO_2 nanoparticles will be the MNMs subject of study. They are currently included in the prioritization lists of relevant reference materials worldwide, defined considering production volume, main market applications and persistence in the environmental compartments.

The preparation and characterization of the MNM dispersions represent key steps to improve the reproducibility and reliability of the toxicity test results, considering the inherent instability of the nanomaterials selected in the aqueous media of the test organisms. This issue is intended to be addresed through the study of the energy delivered to the MNMs using different dispersion processes, the analysis of the methods employed to determine the MNM concentrations, and the addition of organic matter to the exposure media. The latter will also allow conducting the tests in environmentally realistic conditions.

Organisms of different trophic levels, selected in terms of cost, ecological relevance, reproducibility and sensitivity, will be tested in the ecotoxicological analysis. It will comprise seawater bacterium *Vibrio fischeri* (UNE-EN ISO 11348-2:2009), and the freshwater crustacean *Daphnia magna* (OECD Test Guideline 202) and microalgae *Pseudokirchneriella subcapitata* (OECD Test Guideline 201).

Chapter 4. Methodology

The general methodology used in the present study is based on four major aspects: the selection of manufactured nanomaterials (MNMs), the dispersion and characterization strategies, and the ecotoxicological assessment:



Additional information on the instrumental techniques mentioned in this Chapter is included in ANNEX 1.

4.1. Selection of manufactured nanomaterials (MNMs)

Multiwalled carbon nanotubes (MWCNTs) and TiO_2 and CeO_2 nanoparticles were selected as subject of study, since they are currently included in the prioritization lists of relevant reference materials worldwide (defined considering production volume, main market applications and persistence in the environmental compartments). The physical descriptions of these MNMs were provided by the manufacturers (Table 4.1).

Table 4.1 Physical descriptions of the MWCNTs studied.

Nanomaterial	Identification	Size/Outer diameter (nm)	Length (nm)	Impurities (metal oxides) (%)
CNT-1	Nanocyl NC7000	6-24	2000-5000	‹5
CNT-2	Arkema Graphistrength C100	10-15	100-10000	‹10
CNT-3	JRCNM04000a	9-18	400-1300	16.2
CNT-4	JRCNM04001a	28-99	1600-6500	18.1
CeO_2	JRCNM02102a	33	-	‹0.1%
TiO_2	JRCNM01003a	25	-	4.1%

4.2. Preparation of the MNM dispersions

The preparation of the MNM dispersions is crucial for improving the reproducibility and reliability of the *in vivo* tests. Nevertheless, the inherent hydrophobicity and tendency to agglomerate of the studied MNMs hinder to obtain reasonable stability and homogeneity of the dispersions.

Sonication processes were conducted to suspend the MNMs in the aquatic environments, using an ultrasonic bath (Sonorex Digitec DT 255/H, BANDELIN) and an ultrasonic homogenizer (VIBRACELL-VCX750, SONICS&MATERIALS) equipped with a standard probe (136 mm length and 13 mm diameter).

In addition, the dispersions were prepared in the presence of synthetic and natural organic matters to enhance their stability during the different exposure tests.

4.3. Characterization of the MNM dispersions

Characterization of the MNM dispersions by DLS (Dynamic Light Scattering), SEM (Scanning Electron Microscopy) and UV/Vis (Ultraviolet-visible) spectroscopy was conducted immediately after their preparation and at the beginning and end of the ecotoxicity tests.

The stability of the MNMs dispersions was assessed by measuring the variation in scattered light intensity and calculated average zeta-sizes as a function of time. For this purpose, Zeta-average diameter (Z_{ave}), polydispersity index (PDI) values, zeta potentials and electrophoretic mobility (EPM) were obtained by light scattering measurements in a Malvern Zetasizer Nano ZS instrument, considering the data generated from 10 repeated measurements.

Electron microscopy was conducted on MNMs dispersions using a ZEISS apparatus (ULTRA PLUS model) and a JEOL apparatus (JSM-7000F model).

UV/Vis spectroscopy was selected to analyze the stability and determine the relative concentrations of MNMs. An UV/Vis/NIR (Ultraviolet-visible-near infrared) spectrophotometer (Lambda 950, PerkinElmer) and quartz cells with 10 mm path length were used for this purpose.

4.4. Ecotoxicity tests

Organisms of different trophic levels, selected in terms of cost, ecological relevance, reproducibility and sensitivity, were involved in the ecotoxicological analysis. It comprised seawater bacterium *Vibrio fischeri* (UNE-EN ISO 11348-2:2009 "Water quality: Determination of the inhibitory effect of water samples on the light emission of *V. fischeri*. Part 2: Method using liquid-dried bacteria"), and the freshwater crustacean *Daphnia magna* (OECD Test Guideline 202 "Daphnia sp., Acute Immobilization Test") and microalgae *Pseudokirchneriella subcapitata* (OECD Test Guideline 201"Algal growth inhibition test").

The algal inhibition test included fluorometric determinations to obtain the chlorophyll-a concentrations. Extracted chlorophyll permitted the estimation of the biomass of the algal cultures in the presence of MNMs, which interfere with measurements of culture density normally made by optical absorbance. The

fluorescence of the samples was determined in arbitrary units on a microplate reader (FLUOstar OPTIMA, BMG-LABTECH,) with an excitation wavelength of 430 nm and a measured emission wavelength of 670 nm. The needed sub-sample volume was 350 µL in 96-well polypropylene black microplates (Greiner Bio One).

Chapter 5. General overview

The research presented herein comprises the current state of the art in the risk assessment of manufactured nanomaterials (MNMs) and each of the research papers published on this issue. In the following paragraphs, a general overview of the study conducted and the advances in knowledge made are briefly recapitulated.

It can be outlined from the introductory chapter that the present work has been aimed at defining test methods to overcome the current limitations of the toxicological assessment of MNMs in aquatic ecosystems.

Contribution 1 has provided a basis for the subsequent studies, focusing on the dispersion methods of multiwalled carbon nanotubes (MWCNTs) in the aqueous media of organisms and the determination of concentrations, which constitute relevant aspects to obtain accurate ecotoxicity results. MWCNTs are produced worldwide on a large-scale and their applications are steadily increasing. UV-visible spectroscopy is one of the most reported techniques in the last ten years to determine quantitatively their concentrations in dispersions. A key factor in this task is the preparation of calibration curves based on dispersions with previously known

concentrations. Some previous studies have not used the same methods (variable sonication processes) to prepare samples for calibration curves and samples for toxicity assessment, which could lead to misleading results in concentration values.

This work has studied whether those different preparation techniques result in the same dispersion state of MWCNTs and UV/Vis absorbance results. The sonication process for calibration dispersions has been carried out using an ultrasonic homogenizer and a 'verification' has been conducted by preparing dispersions with an ultrasonic bath. In addition, the delivered acoustic energy supplied by the ultrasonicators to the MWCNTs during the preparation of dispersions has been calculated and optimized. This aspect had not been considered in the literature in some cases, resulting in significant damage to the MWCNTs and altering their behavior within the context of toxicological testing. Furthermore, considering that variations in the wavelengths selected for absorbance measurements have also been observed in the literature, a procedure to select an appropriate wavelength for each type of MWCNT has been proposed (Figure 5.1). After the optimization of the dispersion parameters, ecotoxicity tests for MWCNTs have been performed on *Vibrio fischeri*. This organism has been selected taking into account that bacteria constitute the lowest organism level and the entrance to the food web in many ecosystems.

The results obtained have demonstrated that UV/Vis absorbance absolutely depends on the dispersion method. Dispersions for calibration curves and for toxicity assessment should be prepared using the same method to achieve the same absorbance results.

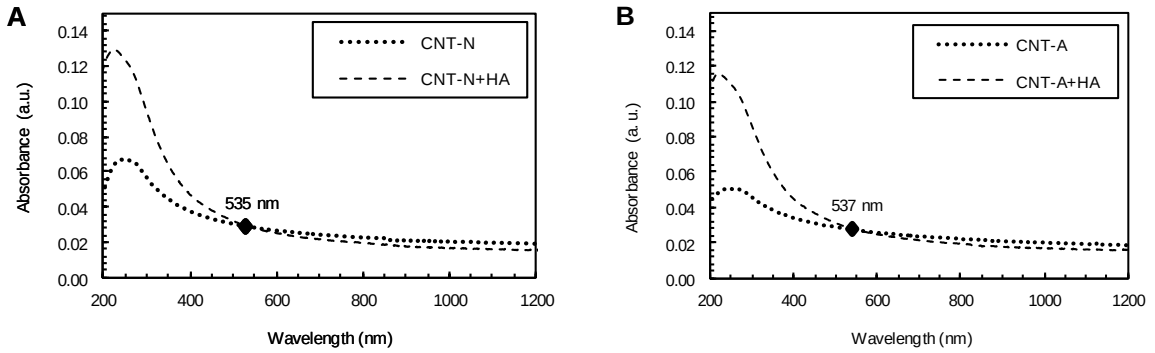


Figure 5.1 Ultraviolet-visible spectra of MWCNT dispersions considering humic acid (dotted lines) and ultrapure water (dashed lines) as a background solution for CNT-N (A) and CNT-A (B), and the measurement wavelengths selected to perform calibration curves and verifications. CNT-N = Nanocyl NC7000; CNT-A = Arkema Graphistrength C100; HA = humic acid.

Dynamic Light Scattering (DLS) technique has been used to determine the agglomerate size of carbon nanotubes and has permitted to observe that the ultrasonic probe is the dispersion method producing an optimization of the energy and the lowest agglomeration rates (Figure 5.2). Therefore, the sonicator probe has been the technique selected to prepare the dispersions for the ecotoxicity assessment. Finally, the improvements in the dispersion performance have also contributed to increase the reliability of the test results and overcome the few and divergent available data for the adverse effects of MWCNTs on *V. fischeri*.

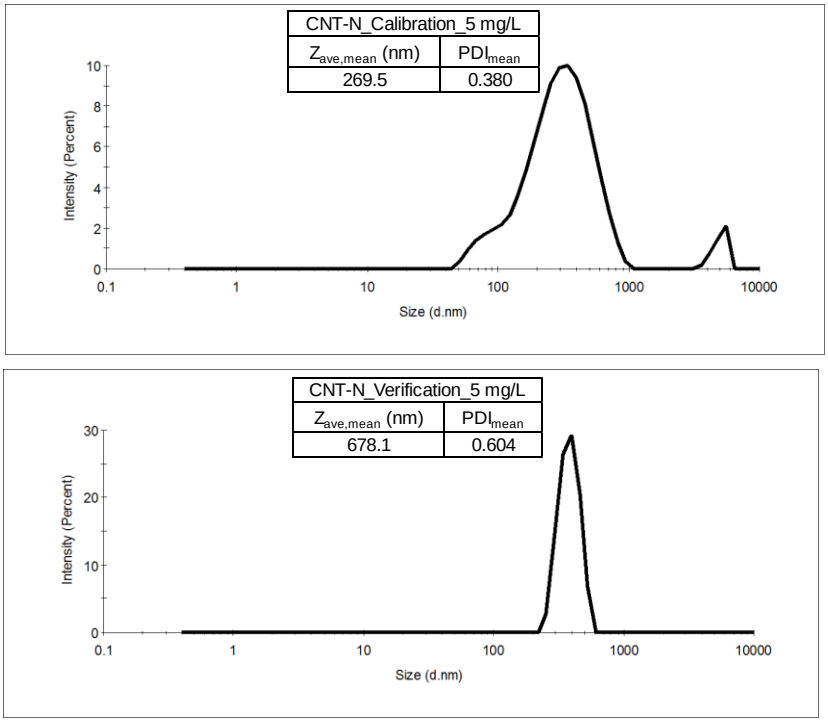


Figure 5.2 Size distributions by intensity of CNT-N nanotubes agglomerates in calibration and verification dispersions.

Once the dispersion methods have been optimized, the research conducted in Contribution 2 has been aimed to compare the agglomeration kinetics and ecotoxicity of MWCNTs in the presence of different solution conditions of aquatic environments, in order to determine the most appropriate to fulfill the current regulation requirements.

Apart from the dispersion methods and techniques to determine concentrations (analyzed in Contribution 1), other factors such as impurities, surface modifications, variable structures and exposure routes of MWCNTs influence the divergent toxicity results that have been published so far. Moreover, their inherent hydrophobicity usually results in agglomeration and settlement behaviors that hinder stability for

their ecotoxicological assessment in aqueous systems. The solution conditions of aquatic environments also determine their bioavailability. Previous studies have analyzed the interactions between MWCNTs and the substances present in natural waters, such as salts containing monovalent and divalent ions as well as natural organic matter (NOM). High ionic strength and low pH induce the colloidal destabilization of MWCNTs, whereas humic substances (the major fraction in NOM) promote their stabilization. Suwannee River NOM (SR-NOM) and Sigma-Aldrich humic acid are the organic substances most extensively studied on this issue. The former presents the key advantage of simulating the real ecosystems in the toxicity assays, unlike laboratory-synthesized humic substances. Many authors have demonstrated the influence of the type of organic matter used in the tests on the adverse effects of MWCNTs on aquatic organisms.

The research conducted has compared the agglomeration kinetics and ecotoxicity of MWCNTs in the presence of the most referenced synthetic and natural organic matters: Sigma Aldrich humic acid and SR-NOM, respectively (Figure 5.3). Inhibitory effects on the key invertebrate organisms for regulatory testing *Daphnia magna* have been studied.

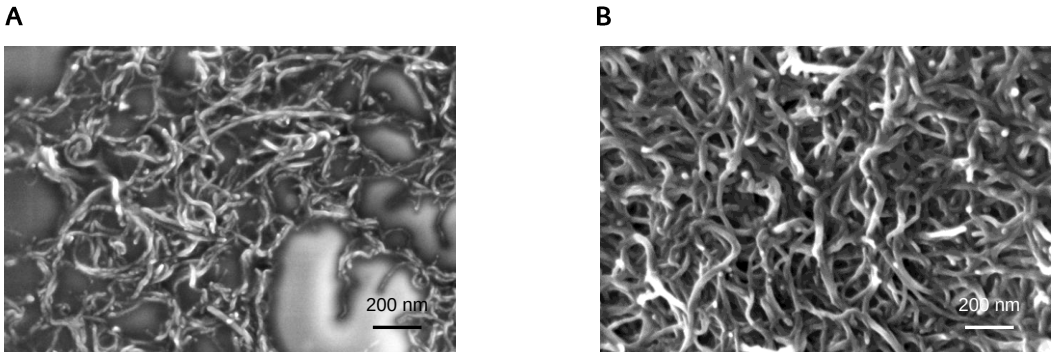


Figure 5.3 Scanning electron microscope images of the 50 mg/L MWCNT dispersions in the presence of SR-NOM (A) and humic acid (B).

The characterization performed by DLS, UV/Vis spectroscopy and scanning electron microscopy (SEM) has indicated that NOM provides an increased stability to the MWCNT dispersions with respect to synthetic organic matter. Sigma-Aldrich humic acid (HA) has appeared to alter the response of the organisms to carbon nanotubes compared with that shown in the presence of SR-NOM (Table 5.1 and Figure 5.4).

Table 5.1 Effective concentration values and lower and upper 95% confidence limits (CL), of MWCNTs dispersions (mg/L) for *Daphnia magna* neonates during 48 h.

Dispersant	Sample	EC20 (95% CL)	EC50 (95% CL)
SR-NOM	CNT-1	4.03 (3.65-4.45)	›50
	CNT-2	2.94 (2.60-3.31)	›50
	CNT-3	ND	ND
	CNT-4	1.08 (0.86-1.35)	27.05 (21.47-34.08)
	SR-NOM	›20	›20
HA	CNT-1	333.15 (315.66-351.60)	››50
	CNT-2	ND	ND
	CNT-3	ND	ND
	CNT-4	ND	ND
	HA	›20	›20

HA = humic acid; ND = not determined; CNT-1 = Nanocyl NC7000; CNT-2 = Arkema Graphistrength C100; CNT-3 = Joint Research Centre-European Comission Repository- JRCNM04000a; CNT-4 = Joint Research Centre-European Comission Repository-JRCNM04001a.

The dispersions prepared with SR-NOM have shown agglomerates of MWCNTs on the body surface of the organisms (Figure 5.4A), and the accumulation of nanotubes on their external surface represents a potential mechanism of toxicity. In the case of HA dispersions, a greater amount of MWCNTs has been observed in the digestive tract of daphnids, given their dark coloration (Figure 5.4B). Nonetheless, the organisms exposed to humic acid alone as background substance (Figure 5.4C) have also shown this coloration. This fact could pose a greater uptake of HA by *D. magna* as a food source and thus explain the reduction in immobilization with respect to MWCNTs dispersed in SR-NOM.

Figure 5.4 Optical microscope images of *Daphnia magna* exposed to the multiwalled carbon nanotube dispersions at the end of the tests.

Furthermore, the results obtained with SR-NOM have allowed observing the important role of the outer diameter and content of impurities of MWCNTs in their stability and ecotoxicity on daphnids. Suwannee River-NOM is considered to be more appropriate for the ecotoxicological assessment of MWCNTs, not only due to the stability provided to the dispersions, but also to its capability of simulating the real conditions in aquatic ecosystems.

Contributions 1 and 2 have presented an optimization of the methodologies to prepare aqueous dispersions for the risk assessment of MWCNTs on organisms representing different trophic levels, such as bacterium (decomposer) and crustacean (primary consumer). These advances have been used to overcome the knowledge gaps on the risk assessment of two of the most extensively metal oxide nanomaterials currently manufactured: TiO_2 and CeO_2 nanoparticles. The data published on their aquatic toxicity are also divergent, which poses a barrier to their current and potential applications. The combination of their physicochemical properties and environmental conditions may result in either their agglomeration or stabilization, determining their bioavailability and toxicity.

Since SR-NOM has been selected as an appropriate and useful stabilizing substance for MWCNTs in Contribution 2, Contribution 3 has been intended to serve as a next step toward the demonstration of its suitability for the ecotoxicological assessment of MNMs. The agglomeration kinetics and ecotoxicity of CeO_2 and TiO_2 NPs towards *Pseudokirchneriella subcapitata* have been analyzed in the presence and absence of SR-NOM. These unicellular green algae have been selected considering their key role in the aquatic ecosystems (primary producer in the food web).

SR-NOM markedly has increased the stability of the NPs in algal medium (Figure 5.5), which has led to a better reproducibility of the toxicity test results. In addition, the agglomeration kinetics observed in the presence of organic matter are similar to that previously reported in various river and groundwaters. Furthermore, SR-NOM has alleviated the adverse effects of NPs on algal growth, completely in the case of TiO_2 NPs and partially in the case of CeO_2 NPs, suggesting a 'camouflage' of toxicity (Figure 5.6). This behavior has been observed also for other algal species and types of natural organic matter in the literature. Thus, SR-NOM can be a representative sample of what is found in many different ecosystems, and the observed 'camouflage' of the effects of CeO_2 and TiO_2 NPs on algal cells might be considered as a natural interaction occurring in their standardized ecotoxicological assessment.

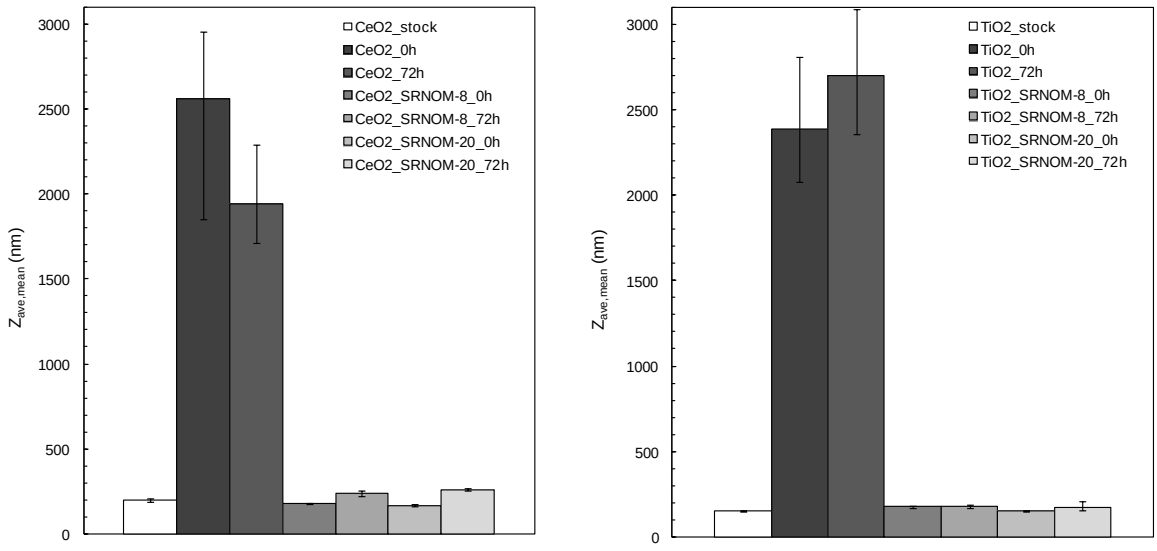


Figure 5.5 Histogram comparisons of Z_{ave} size of the NP stock dispersions, and in the presence/absence of 8 and 20 mg/L SR-NOM, at the beginning and end of the exposures (0 h and 72 h, respectively). Error bars represent standard deviation (n=3).

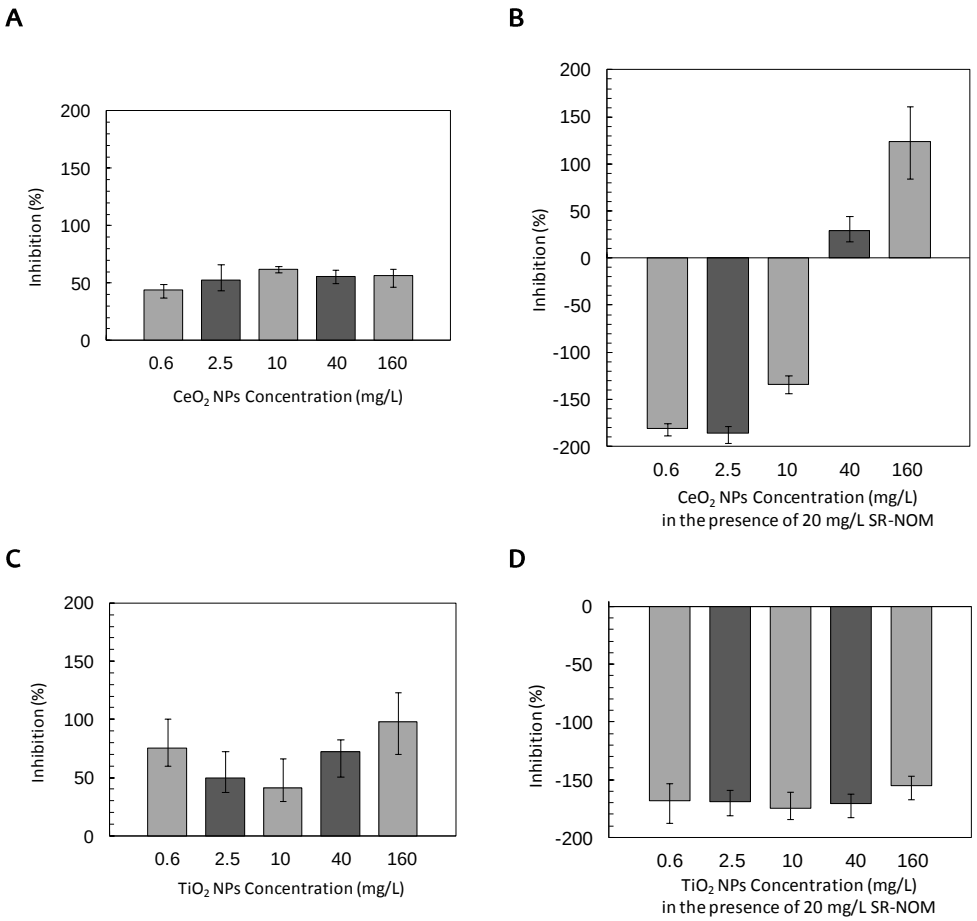


Figure 5.6 Histogram comparisons of percent inhibition in average specific growth rates of *Pseudokirchneriella subcapitata* exposed to CeO_2 and TiO_2 NPs in the absence of SR-NOM (A,C), and in the presence of 20 mg/L SR-NOM (B,D), during 72 h. Error bars represent standard deviation (n=3).

The major contributions made by the present study to the toxicological assessment of MNMs in aquatic ecosystems comprise: the optimization of the MNM dispersion methods in the aqueous media of the test organisms and the selection of a reference natural organic matter to conduct the exposures in environmentally realistic conditions. These achievements have also led to a better reproducibility of the toxicity test results.

Chapter 6. Results and discussion

6.1. Contribution 1

Ecotoxicity of multiwalled carbon nanotubes: standardization of the dispersion methods and concentration measurements

Environmental Toxicology and Chemistry 2015, 34: 1854-1862

Contents

Abstract:

There are currently a variety of applications for multiwalled carbon nanotubes (MWCNTs), but considerable concerns exist regarding their release into the environment. Their potential accumulation by aquatic organisms could lead to transfer throughout food chains. Considering the divergences in experimental data published on the ecotoxicity of carbon nanotubes, further research is required. The dispersion of MWCNTs in aqueous culturing media of organisms as well as the determination of concentrations are relevant aspects to obtain accurate ecotoxicity results. Ultraviolet-visible spectroscopy is one of the most reported techniques to analyze concentration quickly and economically, but the methodologies to prepare dispersions and selecting the wavelengths for ultraviolet-visible measurements have not yet been clearly defined. The present study demonstrates that dispersion procedures influence absorbance, and an approach to determine the most appropriate measurement wavelength is proposed. Ecotoxicity tests with MWCNTs were performed on Vibrio fischeri *bacteria, and divergences in the results were observed with respect to those previously reported. The present study contributes to the attempt to overcome the lack of standardization in the environmental assessment of MWCNTs.*

Keywords: Ecotoxicity; Multiwalled carbon nanotube; Humic acid; Sonication; Ultraviolet-visible spectroscopy.

6.1.1. Introduction

Nanotechnology is playing a key role in the development of goods and services around the world and fostering the competitiveness of industries in the knowledge economy. Within nanomaterials, the unique physical, chemical, electrical, and mechanical properties of carbon nanotubes (CNTs) are promoting the increase in the number of applications in different fields (e.g., chemistry, electronics, energy, materials science, medicine).[1,2] Large-scale production and applications of CNTs are steadily increasing. Thus, there are considerable concerns over their inevitable release into the environment and human exposure to them because their accumulation by aquatic organisms could lead to transfer throughout food chains.[3-5] In addition, the limited understanding of the environmental, health, and safety aspects of CNTs poses a threat to their potential applications, considering that experimental data related to their toxicity at different levels have been published[6,7] and that the results are often divergent. This inconsistency could be a consequence of factors such as impurities, surface modifications, structure, and exposure routes.[4] Therefore, more attention to toxicology research on them is required to achieve a systematic understanding of their real toxicity.

The number of industrial-scale facilities for the relatively low-cost production of multiwalled CNTs (MWCNTs) is growing steadily,[8,9] and their release into the

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[1] Terrones M. **2003**. Science and technology of the twenty-first century: Synthesis, properties, and applications of carbon nanotubes. *Annu Rev Mater Res* 33:419-501.

[2] Heister E, Brunner EW, Dieckmann GR, Jurewicz I, Dalton AB. **2013**. Are carbon nanotubes a natural solution? Applications in biology and medicine. *ACS Appl Mater Inter* 5:1870-1891.

[3] Firme CP, Bandaru PR. **2010**. Toxicity issues in the application of carbon nanotubes to biological systems. Nanomedicine Nanotechnology Biology and Medicine 6:245-256. Bennett SW, Adeleye A, Ji Z, Keller AA. 2013. Stability, metal leaching, photoactivity and toxicity in freshwater systems of commercial single wall carbon nanotubes. *Water Res* 47:4074-4085.

[4] Liu Y, Zhao Y, Sun B, Chen C. **2013**. Understanding the toxicity of carbon nanotubes. *Accounts Chem Res* 46:702–713.

[5] Bennett SW, Adeleye A, Ji Z, Keller AA. **2013**. Stability, metal leaching, photoactivity and toxicity in freshwater systems of commercial single wall carbon nanotubes. *Water Res* 47:4074-4085.

[6] Zhao X, Liu R. **2012**. Recent progress and perspectives on the toxicity of carbon nanotubes at organism, organ, cell, and biomacromolecule levels. *Environ Int* 40:244-255.

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[8] Ray PC, Yu H, Fu PP. **2009**. Toxicity and environmental risks of nanomaterials: Challenges and future needs. *J Environ Sci Health* 27: 1-35.

[9] Donaldson K, Aitken R, Tran L, Stone V, Duffin R, Forrest G, Alexander A. **2006**. Carbon nanotubes: A review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicol Sci* 92:5-22.

environment is foreseen to be greater than that of single-walled CNTs (SWCNTs). Thus, research on MWCNT toxicity is considered to be more imperative.

A relevant issue in ecotoxicity studies is the solubility of toxicants in aqueous culturing media. An important obstacle must be faced regarding this issue because CNTs exhibit a hydrophobic nature and a tendency to form agglomerates, which hinders the preparation of stable dispersions in water.[10] Many effective methods, both physical and chemical, have been proposed to disperse CNTs in aqueous solutions, such as stirring, sonication, and addition of surfactants.[11] Nevertheless, the use of these treatments affects the inherent properties of CNTs[12] and, therefore, the interactions they might have with living organisms.[13-15] Because of this, minimizing the effect of these physicochemical treatments on the CNT characteristics becomes necessary. With regard to physical methods, sonication time, frequency, and power have been proven to modify the attributes of nanotubes, such as length and, hence, toxicity.[16,17] Therefore, the reduced energy delivered by sonication baths can be thought to be more appropriate than that of sonication probes. In relation to chemical treatments, selection of biocompatible dispersants is required to avoid the alteration of the toxicity effect of nanotubes. Several surfactants did not show toxicity in living organisms at low concentration levels.[18] However, the most suitable dispersants for ecotoxicity studies are those present naturally in environmental

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[10] Kim SW, Kim T, Kim YS, Choi HS, Lim HJ, Yang SJ, Park CR. **2012**. Surface modifications for the effective dispersion of carbon nanotubes in solvents and polymers. *Carbon* 50:3-33.

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[12] Petersen EJ, Henry TB. **2012**. Methodological considerations for testing the ecotoxicity of carbon nanotubes and fullerenes: Review. *Environ Toxicol Chem* 31:60-72.

[13] Handy RD, Cornelis G, Fernandes T, Tsyusko O, Decho A, Sabo- Attwood T, Metcalfe C, Steevens JA, Klaine SJ, Koelmans AA, Horne N. **2012**. Ecotoxicity test methods for engineered nanomaterials: Practical experiences and recommendations from the bench. *Environ Toxicol Chem* 31:15-31.

[14] Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TS, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR. **2008**. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environ Toxicol Chem* 27:1825-1851.

[15] Li M, Huang CP. **2011**. The responses of Ceriodaphnia dubia toward multi-walled carbon nanotubes: Effect of physical-chemical treatment. *Carbon* 49:1672-1679.

[16] Vichchulada P, Cauble MA, Abdi EA, Obi EI, Zhang Q, Lay MD. **2010**. Sonication power for length control of single-walled carbon nanotubes in aqueous suspensions used for 2-dimensional network formation. *J Phys Chem C* 114:12490-12495.

[17] Johnston HJ, Hutchison GR, Christensen FM, Peters S, Hankin S, Aschberger K, Stone V. **2010**. A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: The contribution of physico-chemical characteristics. *Nanotoxicology* 4:207-246.

[18] Kim JS, Song KS, Lee JH, Yu IJ. **2011**. Evaluation of biocompatible dispersants for carbon nanotube toxicity tests. *Arch Toxicol* 85:1499-1508.

media, such as natural organic matter (NOM) and its major component, humic acid,[19,20] to reproduce realistic environmental conditions in assays.

Once the dispersion procedure has been selected, determining CNT concentrations in dispersions is a critical issue to obtain accurate toxicity values. Different techniques are currently available to estimate the dispersion state and even stability of CNTs (conventional microscopy including optical microscopy, atomic force microscopy, scanning electron microscopy, and transmission electron microscopy; dynamic light scattering; and zeta-potential measurements).[10] However, those methods are, in most cases, qualitative, and the effect of dispersion cannot be evaluated precisely. In addition, photoluminescence and ultraviolet-visible (UV-visible) spectroscopies have been used to determine quantitatively CNT concentrations. In fact, UV-visible is one of the most reported techniques in the last ten years, given its rapidity, its low cost, and the possibility of concentration measurements of both SWCNT and MWCNT dispersions.[18,21,22] However, UV-visible absorbance poses some challenges that have not been solved clearly to date. A key factor in the determination of concentrations by UV-visible spectroscopy is the preparation of calibration curves, based on dispersions with previously known concentrations.[20] Some studies on this issue do not use exactly the same methods (variable sonication processes) to prepare samples for calibration curves and samples for toxicity assessment.[5,23,24] This fact could lead to misleading results in concentration values because different parameters or preparation techniques result in different dispersion states.[11,25] Furthermore, variations in the wavelengths selected for absorbance measurements are observed. Previous studies have shown that

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[19] Wang P, Shi Q, Liang H, Steuerman DW, Stucky GD, Keller AA. **2008**. Enhanced environmental mobility of carbon nanotubes in the presence of humic acid and their removal from aqueous solution. *Small* 4:2166-2170.

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[24] Di Crescenzo A, Demurtas D, Renzetti A, Siani G, De Maria P, Meneghetti M, Fontana A. **2009**. Disaggregation of single-walled carbon nanotubes (SWNTs) promoted by the ionic liquid-based surfactant 1-hexadecyl-3-vinyl-imidazolium bromide in aqueous solution. *Soft Matter* 5:62-66.

[25] Kennedy AJ, Gunter JC, Chappell MA, Goss JD, Hull MS, Kirgan RA, Steevens JA. **2009**. Influence of nanotube preparation in aquatic bioassays. *Environ Toxicol Chem* 28:1930-1938.

absorbance peaks, achieved at established wavelengths, are linearly correlated with the MWCNT concentration.[20,24] Measurement wavelengths reported are 800 nm,[18,20,23,26] 600 nm,[27,28] 530 nm,[29] 500 nm,[21,30] 298 nm,[25] and 260 nm.[11]

The present study focused on making progress in the field of MWCNT ecotoxicology by improving the accuracy of toxicity assessments. The first objective was to analyze the adequacy of UV-visible spectroscopy for the preparation of calibration curves to determine the concentration of MWCNTs in dispersions. Because some studies on this issue use different sonication processes to prepare samples for calibration curves and toxicity assessment, the present investigates whether those different techniques produce the same UV-visible absorbance results. Furthermore, considering that variations in the wavelengths selected for absorbance measurements are observed in previously mentioned works, we propose a procedure to select an appropriate wavelength for each type of MWCNT. After optimization of the dispersion parameters, ecotoxicity tests for MWCNTs were performed on *Vibrio fischeri* bacteria. This aquatic organism was selected taking into account that bacteria constitute the lowest organism level and the entrance to the food web in many ecosystems. The ecotoxicity data obtained should be considered in terms of reliability because the selection of the most appropriate dispersion methods permits standardization of the study of the environmental effects of MWCNTs.

6.1.2. Materials and Methods

6.1.2.1. Materials for dispersions

Two different commercial MWCNTs, NC7000 (Nanocyl), referred as CNT-N, and Arkema Graphistrength C100 (Arkema), referred as CNT-A, were used as received from the manufacturers. Both were produced via catalytic chemical vapor

[26] Hyung H, Kim JH. **2008**. Natural organic matter (NOM) adsorption to multi-walled carbon nanotubes: Effect of NOM characteristics and water quality parameters. *Environ Sci Technol* 42:4416-4421.
[27] Bai Y, Park IS, Lee SJ, Bae TS, Watari F, Uo M, Lee MH. **2011**. Aqueous dispersion of surfactant-modified multiwalled carbon nanotubes and their application as an antibacterial agent. *Carbon* 49:3663-3671.
[28] Chappell MA, George AJ, Dontsova KM, Porter BE, Price CL, Zhou P, Morikawa E, Kennedy AJ, Steevens JA. **2009**. Surfactive stabilization of multi-walled carbon nanotube dispersions with dissolved humic substances. *Environ Pollut* 157:1081-1087.
[29] Marsh DH, Rance GA, Zaka MH, Whitby RJ, Khlobystov AN. **2007**. Comparison of the stability of multiwalled carbon nanotube dispersions in water. *Phys Chem Chem Phys* 9:5490-5496.
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deposition. Some relevant differences were observed in their physical descriptions (outer diameter, length, and percentage of impurities), which were provided by the manufacturers (Table 6.1.1).

Table 6.1.1 Physical descriptions of the MWCNTs studied.

MWCNT type	Description	Outer diameter (nm)	Inner diameter (nm)	Length (nm)	Purity (%)	Impurities (metal oxides) (%)	Surface Area (m^2/g)
Nanocyl NC7000 (CNT-N)	CCVD multiwall carbon nanotubes	6-24	2-9	2000-5000	›95	‹5	250-300
Arkema Graphistrength C100 (CNT-A)	CCVD multiwall carbon nanotubes	10-15	-	100-10000	›90	‹10	-

CCVD = Catalytic chemical vapor deposition.

Scanning electron microscopy was performed directly on dry CNT powder, using a Zeiss apparatus (Ultra Plus model) with a magnification of 48 000× (see Figure 6.1.1).

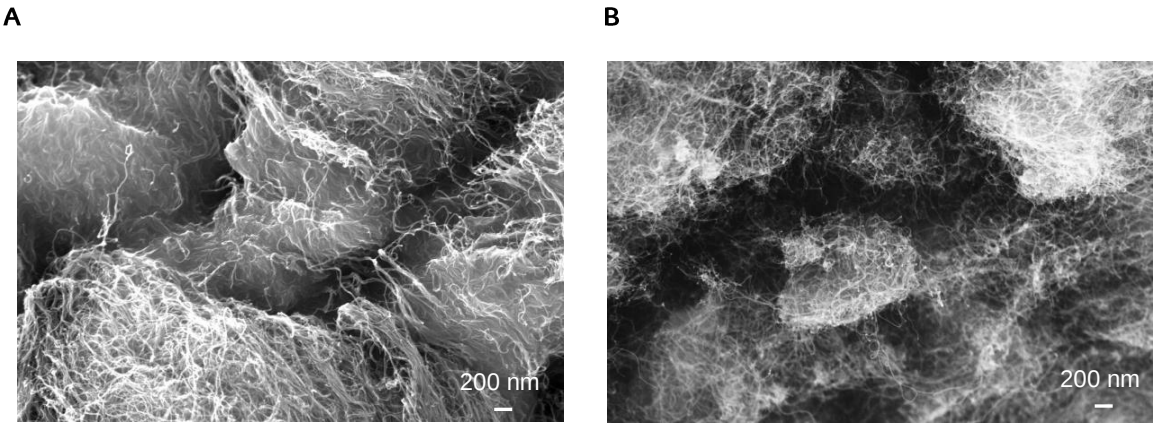

Figure 6.1.1 Scanning electron microscopic images of multiwalled carbon nanotubes: (A) CNT-N and (B) CNT-A. Magnification 48 000×. CNT = carbon nanotube.

Humic acid was purchased from Sigma-Aldrich Química and used without any further purification. Ultrapure water (MilliQ) was produced using a water filtration system from Millipore Iberica to prepare all the dispersions.

6.1.2.2. Dispersion preparation and characterization

Dispersions for calibration curves. Humic acid was selected as the model NOM to prepare the dispersions. The concentrations of humic acid must produce the dispersion of the required amount of MWCNTs. At the same time, these concentrations must be nontoxic to avoid alteration of the ecotoxicity of nanotubes. To ensure the appropriate experimental concentrations of MWCNTs, dispersion tests were performed. The results showed that 30 mg/L of CNTs could be dispersed in 100-mg/L humic acid solutions. Concentrations of 100 mg/L humic acid were experimentally observed to cause no inhibition on *V. fischeri* bacteria, and therefore they were selected to prepare dispersions.

Humic acid solutions were prepared by adding 100 mg/L humic acid into ultrapure water. They were mixed constantly for 48 h at 20±2 °C by means of magnetic stirring, as previously reported.[7,26] This time was sufficient to achieve complete dissolution of humic acid. Thus, further centrifugation or filtration steps to obtain the supernatants were not necessary.

The sonication process for calibration dispersions was carried out as previously described using an ultrasonic homogenizer[31] (Vibracell-VCX750; Sonics & Materials with a standard probe (136 mm length, 13 mm diameter), at an operating frequency of 20 kHz, pulsing operating mode of 1 s on/1 s off, and output power fixed at 750 W at 60% amplitude. Dispersions were prepared by mixing the corresponding amount of MWCNTs with 25-mL of 100-mg/L humic acid solution in 200-mL glass beakers and sonicating for 2.5 min. Sonication was repeated 3 times more, adding 25 mL of humic acid solution at each stage, until the volume was adjusted to achieve a CNT concentration of 30 mg/L. The beaker was held in an ice bath during sonication to prevent a rise in the temperature of the sample and covered with Parafilm (plastic paraffin film) to avoid evaporation.

Calibration standards were made by diluting the 30-mg/L dispersions with 100-mg/L humic acid solution, obtaining 12 more levels: 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, 2.5 mg/L, 2 mg/L, 1.5 mg/L, 1 mg/L, 0.5 mg/L, 0.25 mg/L, and 0.1 mg/L.

Dispersions for verification of calibration curves. The dispersions for verification of calibration curves were also prepared with 100-mg/L humic acid solutions. Three different concentrations in the same range as the calibration dispersions were

[31] Edgington AJ, Roberts AP, Taylor LM, Alloy MM, Reppert J, Rao AM, Mao J, Klaine SJ. **2010**. The influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. Environ *Toxicol Chem* 29:2511-2518.

selected to prepare: 2.5 mg/L, 5 mg/L, and 10 mg/L. These concentrations were high enough to avoid accuracy errors in weighing CNTs but not too high to obtain stable dispersions, according to previously described methods.[23,25] The sonication process was carried out with an ultrasonic bath (Sonorex Digitec DT 255/H) at an operating frequency of 35 kHz and 160 W output power, filling the bath with cool water (15 °C) at the same level as that inside the sample bottles. Dispersions were prepared by mixing the corresponding amount of MWCNTs with 50 mL of 100-mg/L humic acid solution in 250-mL glass flasks and sonicating for 15 min. Sonication was repeated 3 times more, adding 50 mL of humic acid solution at each stage, until the volume was adjusted to achieve the required CNT concentrations for verification. Three flasks were sonicated at the same time in each experiment to ensure the same level of energy received by the dispersions.

Calculation of the total amount of energy delivered by the sonication methods from calorimetry. Given the importance of the dispersion techniques in the present study, the delivered acoustic energy supplied by the ultrasonicators was calculated using the calorimetric method described by Taurozzi et al.[32] A study on this subject[11] has demonstrated that there is a minimum energy required to disperse the optimum amount of MWCNTs in aqueous solution and that their dispersion behavior is also determined by parameters such as the concentration of CNTs and the ratio of CNTs to dispersant. Taking into account the results obtained in that work and the concentrations used in the present study, we established that the total amount of energy delivered to the dispersions prepared should not be higher than 30 kJ to prevent damaging and cutting effects on CNTs.

The acoustic powers delivered by sonicator probe and bath were calculated in a similar manner. A 600 mL borosilicate glass beaker was filled with 500 mL thermally equilibrated MilliQ water. Its temperature and mass were measured with an uncertainty of ±0.1 ºC and ±0.1 g, respectively. In the case of the ultrasonic probe, the 600 mL beaker was placed in the sonicator chamber and the tip was immersed to a position 2.5 cm below the liquid surface. The temperature probe was mounted (using a clamp) at 2.5 cm depth and 1 cm away from the sonicator probe. The sonicator output selected was 60% amplitude, operating in continuous mode. The temperature increase of the water was recorded for 5 minutes with a time resolution of 15 seconds. Sonicator bath was filled with deionized water at the same level as the

[32] Taurozzi JS, Hackley VA, Wiesner MR. **2011**. Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment-Issues and recommendations. *Nanotoxicology* 5:711-729.

one inside the 600 mL beaker and the temperature probe was mounted (using a clamp) at 2.5 cm depth in the center of the beaker. The sonicator operated in continuous mode and the water temperature increase was recorded for 60 minutes with a time-resolution of 2 minutes.

Calculation of the delivered acoustic energy was performed obtaining the best linear fit (R^2›0.990) between the measured temperature and time using least squares regression. The effective delivered power was determined using the Equation 6.1.1:

$$P = \frac{\mathrm{d}T}{\mathrm{d}t} M C_p \qquad (6.1.1)$$

where *P* is the delivered acoustic power (W), *dT/dt* is the slope of the regression curve, *M* is the mass of liquid (g), and C_p is the specific heat of the liquid ($J \cdot g^{-1} \cdot {}^{o}C^{-1}$).

The effective delivered acoustic power (*P*) was 42.26 W for sonicator probe and 7.28 W for sonicator bath. The linear fits between the measured temperature as function of time using least squares regression are represented in Figure 6.1.2.

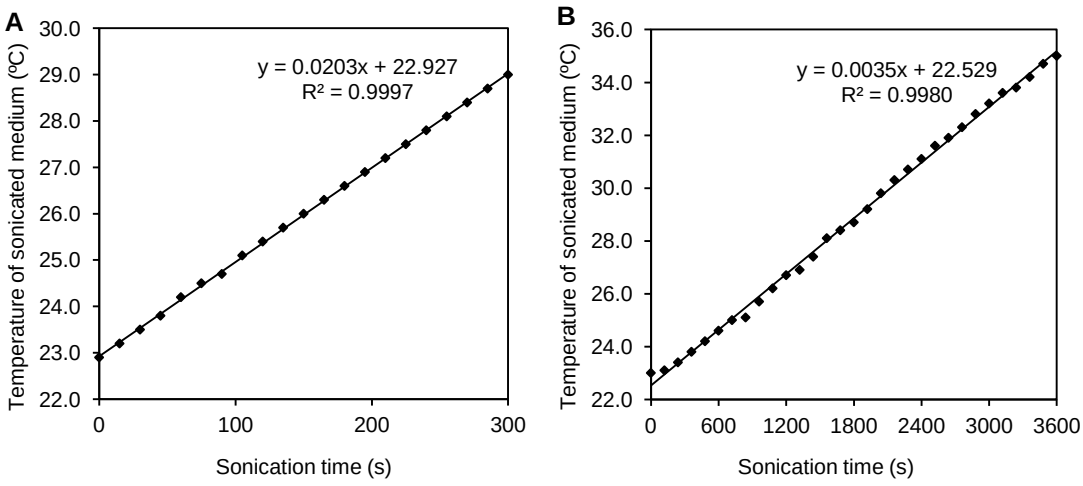

Figure 6.1.2 Linear fits between the measured temperature as function of time sonicator probe (A) and bath (B).

The total amount of energy delivered (Equation 6.1.2) was obtained considering the applied power and also the total amount of time that the water is subjected to the ultrasonic treatment:

$$E = P \times t \qquad (6.1.2)$$

where *E* is the total amount of energy (J), *P* is the delivered acoustic power (W) and *t* is the total amount of time (s).

It is important to consider that the actual volumes and temperatures of MWCNTs dispersions were different from that used in the calculation of the energy delivered by the sonication methods. This aspect is noted in the calorimetric method, which is simply intended to allow the reporting and transference of sonication power levels between users, but not to measure the actual fraction of power utilized for powder disruption under specific dispersion conditions.

Considering the sonication times selected, the total amount of energy delivered (*E*) was 12.68 kJ for the sonicator probe and 26.20 kJ for the sonicator bath. Because a similar energy for both sonication methods was required to obtain comparable results, the sonication time for the sonicator probe was duplicated for the preparation of dispersions (from 5 min to 10 min), and the total amount of energy delivered was finally 25.35 kJ. These values were in accordance with the maximum specified above to prevent harmful effects on CNTs.

Dispersion characterization. Two factors affect the UV-visible absorbance ability of MWCNTs: their intrinsic properties and the agglomeration rate. If the size of the agglomerates is comparable to the wavelength of the light, the intrinsic properties are the main influencing factor. If the size of the MWCNT agglomerates is much larger than the wavelength, the agglomeration rate is the main influencing factor.[21] Therefore, absorbance peaks vary depending on both the features of the CNTs and the methods employed to prepare dispersions. Thus, a spectral analysis is always necessary to check the absorbance maxima of the studied nanotubes.

Absorbance spectra for 30-mg/L dispersions were obtained immediately after sonication, and a spectral analysis of the humic acid solution was performed to check that it did not alter the baseline of MWCNT absorbance spectra.[20] Absorbance spectra were obtained using a UV-visible spectrophotometer (Lambda 950; PerkinElmer) and quartz cells with a 0.2-mm path length. Although the initial operating range of the spectrophotometer was 200 nm to 2000 nm, because no remarkable changes in the spectra were appreciated over 1200 nm, the final wavelength range selected in the present study was 200 nm to 1200 nm, considering also the data reported on this issue.[11] The wavelength for calibration curve measurements was selected considering the absorbance spectra of MWCNTs and humic acid (see Results and Discussion). Measurements for calibration curves were conducted using a Jenway 6300 spectrophotometer, which provided more speed to obtain absorbance at a specific wavelength. This equipment operates at 320 nm to 1000 nm wavelength with 10-mm path length quartz cells.

Prior to the UV-visible absorbance measurements, the dispersions were characterized with the aim of analyzing their stability by size distributions and rate of agglomeration. Thus, these properties could be compared for both types of dispersions, and it could be checked whether the sonication parameters selected (time and amplitude) were appropriate. The Z-average diameter (Z_{ave}) and polydispersity index were obtained by dynamic light scattering measurements in a Malvern Zetasizer Nano ZS instrument, considering the data generated from 10 repeated measurements.

6.1.2.3. Selection of the dispersion method and ecotoxicity tests

Based on the results of the dispersion characterization, the most appropriate sonication method to prepare dispersions for ecotoxicity tests was determined. To check the effective deagglomeration efficiency of MWCNTs in these dispersions, dynamic light scattering measurements were carried out immediately after their preparation. Moreover, the concentrations of CNTs were measured by UV-visible absorbance to check whether these values corresponded to those of calibration curves.

Vibrio fischeri bacteria were selected to carry out the ecotoxicity tests, considering that very few studies have reported data of CNT toxicity on this microorganism.[33,34] The assays were performed on LUMIStox 300 photometer controlled by LUMISsoft IV software (Dr. Lange), according to UNE-EN ISO 11348-2:2009.[35] *Vibrio fischeri* produces light as a by-product of its cellular respiration, and the assay results were the toxicant effective concentrations causing 20% (EC20) and 50% (EC50) inhibition in light emission. The exposure time between dilution rows of the samples and bacteria was 30 min, and the reference substance used was $K_2Cr_2O_7$ (Sigma-Aldrich Química). Three independent tests were performed, and standard deviation values for EC20 and EC50 were calculated.

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[33] Blaise C, Gagne F, Fe JF, Canada E, Street M. **2008**. Ecotoxicity of selected nano-materials to aquatic organisms. *Environ Toxicol* 23:591-598.

[34] Zheng H, Liu L, Lu Y, Long Y, Wang L, Ho KP, Wong KY. **2010**. Rapid determination of nanotoxicity using luminous bacteria. *Anal Sci* 26:125-128.

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6.1.3. Results and Discussion

6.1.3.1. Selection of the measurement wavelength

As mentioned in *Materials and Methods*, the spectral analysis was intended to determine MWCNT absorbance peaks and whether there was any alteration in their spectra as a result of the presence of humic acid in dispersions, with the aim of selecting the measurement wavelengths for each CNT. For this purpose, the spectra of 30 mg/L MWCNT dispersions were obtained in 2 different ways: 1) considering the 100-mg/L humic acid solution effect, taking it as a background substance, and subtracting its absorbance by the "autozero" function of the spectrophotometer and 2) measuring absorbance of dispersions by taking ultrapure water as a background solution. Thus, we could analyze whether the absorbance of both humic acid and MWCNTs was additive, as previously reported,[5] and whether this fact was noticed along the whole spectrum. Figure 6.1.3 shows absorbance peaks of humic acid and MWCNTs in arbitrary units (a.u.).

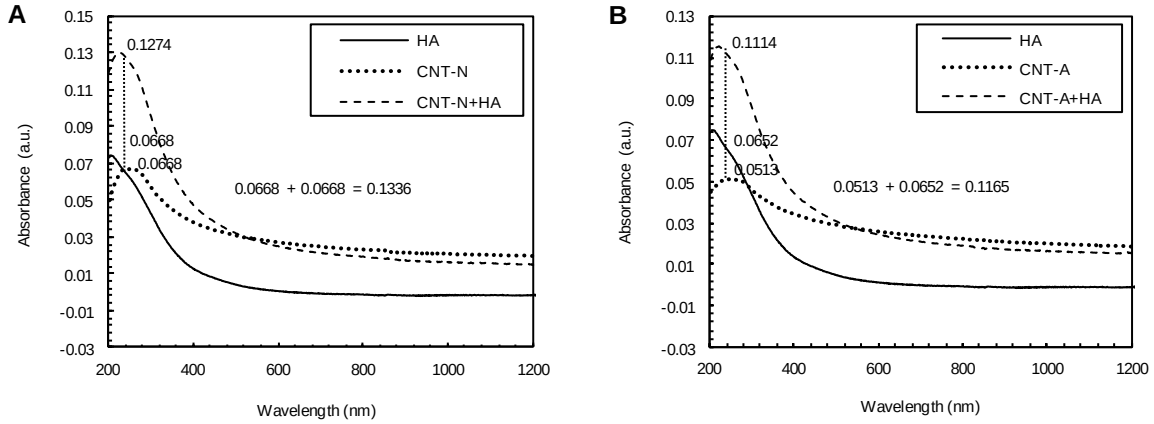

Figure 6.1.3 Ultraviolet-visible spectra of humic acid solution (continuous lines), multiwalled carbon nanotube dispersions considering humic acid as a background solution (dotted lines), and multiwalled carbon nanotube dispersions considering ultrapure water as a background solution (dashed lines) for CNT-N (A) and CNT-A (B). CNT = carbon nanotube; HA = humic acid.

Absorbance peaks of humic acid and MWCNTs were observed at similar wavelengths. The humic acid absorbance maximum was achieved at 206 nm (Figure 6.1.3, continuous line), and those for CNT-N and CNT-A (dotted lines) were at 240 nm and 241 nm, respectively. Considering the spectra of dispersions with humic acid (dashed lines), a shift to the left was observed at absorbance peaks, decreasing to

227 nm and 222 nm. Although these maxima were higher than those for CNT-N and CNT-A without humic acid, humic acid involved alteration of the UV-visible absorbance of MWCNT dispersions. The absorbances of CNTs and humic acid were not fully additive because a deviation of the calculated values was obtained with respect to theoretical ones at the CNT peak maxima (see Figure 6.1.3). Because of this, the wavelengths for calibration curves were moved to other spectral values, different from the absorbance peaks. Dispersions with humic acid and those in which its effect was subtracted overlapped their absorbance spectra in a specific wavelength (Figure 6.1.4). This fact could imply that, at this wavelength, the humic acid effect was nonexistent. Those wavelengths were 535 nm for CNT-N and 537 nm for CNT-A, similar to the previously reported wavelength of 530 nm.[29] Hence, they were selected to perform calibration curve measurements and the corresponding verifications.

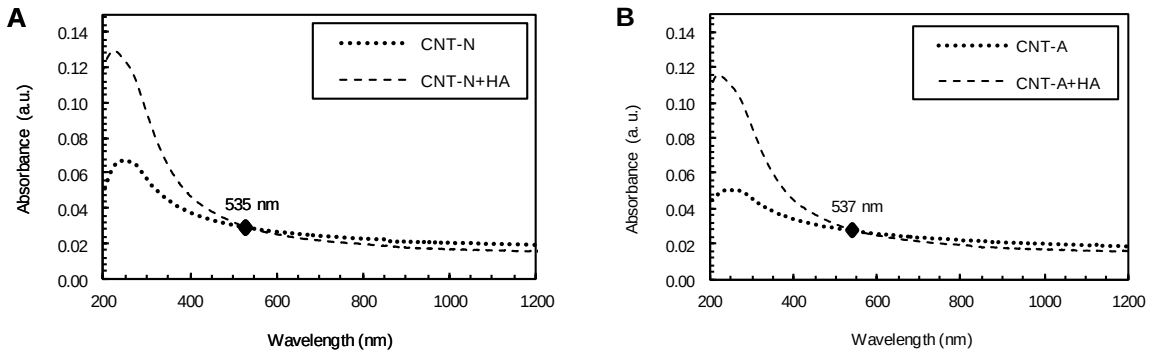

Figure 6.1.4 Ultraviolet-visible spectra of multiwalled carbon nanotube dispersions considering humic acid as a background solution (dotted lines) and multiwalled carbon nanotube dispersions considering ultrapure water as a background solution (dashed lines) for CNT-N (A) and CNT-A (B), and the measurement wavelengths selected to perform calibration curves and verifications. CNT = carbon nanotube; HA = humic acid; a.u. = arbitrary unit.

6.1.3.2. Calibration curves and verification

Taking into account the wavelengths selected to carry out measurements, absorbance values were obtained for each dilution level and type of CNT studied (Table 6.1.2). Dispersions were measured taking ultrapure water as a background substance.

Table 6.1.2 UV/Vis absorbance values to perform calibration curves, for each dilution level and type of MWCNT.

Concentration (mg/L)	CNT-N Absorbance (535 nm)	CNT-A Absorbance (537 nm)
30	-	-
25	1.754	-
20	1.440	-
15	1.045	1.685
10	0.749	1.104
5	0.344	0.567
2.5	0.252	0.283
2	0.153	0.236
1.5	0.107	0.186
1	0.080	0.126
0.5	0.039	0.071
0.25	0.020	0.040
0.1	0.000	0.015

Previous studies have reported calibration curves with absorbances that range from 0.1 a.u. to 1.1 a.u.[19] and from 0.1 a.u. to 0.5 a.u.[21] The obtained absorbance values (Figure 6.1.5) were in the same range as those previously reported. Variations were caused by the different ultrasonic treatments used, the types and concentrations of CNTs and dispersants, and the spectrophotometers employed to perform absorbance measurements. Furthermore, considering that the absorbance range of the spectrophotometer was between −0.300 and 1.999, it was not possible to obtain absorbance values for the highest concentrations (30 mg/L for CNT-N and 20 mg/L, 25 mg/L, and 30 mg/ L for CNT-A; Table 6.1.2; Figure 6.1.5).

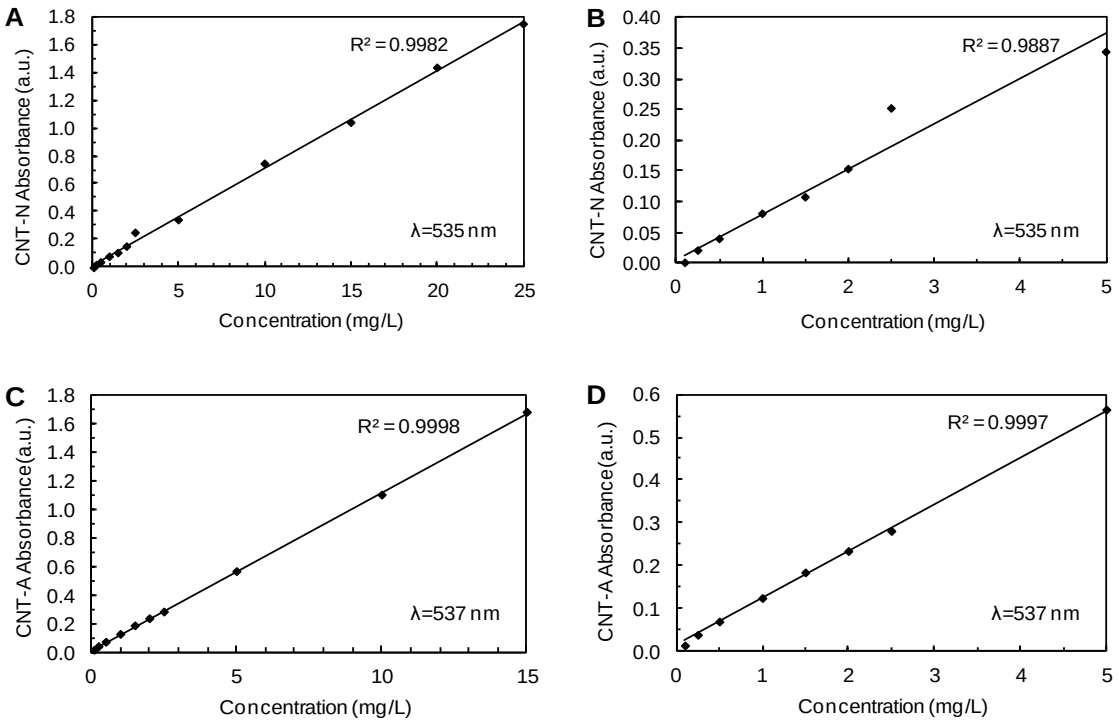

Figure 6.1.5 Calibration curves obtained from absorbance of carbon nanotube dispersions in different ranges of dilution levels, from 0.1 mg/L to 30 mg/L (A,C) and from 0.1 mg/L to 10 mg/L (B,D). Straight lines are linear least-squares fit to the data. CNT = carbon nanotube; a.u. = arbitrary unit.

As explained in *Materials and Methods*, absorbance values for calibration were only verified at specific concentrations: 2.5 mg/L, 5 mg/L, and 10 mg/L. Table 6.1.3 includes absorbance values obtained for these dispersions at the same wavelengths used to prepare calibration curves and taking ultrapure water as the background substance.

Table 6.1.3 Verification and calibration curve values obtained by ultraviolet-visible absorbance for multiwalled carbon nanotube dispersions.

Concentration (mg/L)	CNT-N Absorbance (535 nm)		CNT-A Absorbance (537 nm)	
	Calibration value	Verification value	Calibration value	Verification value
2.5	0.252	0.151	0.283	0.133
5	0.344	0.167	0.567	0.227
10	0.749	0.235	1.104	0.448

CNT = carbon nanotube

For both CNT-N and CNT-A, the verification values showed considerable differences with respect to calibration values. Verification dispersions did not achieve the

absorbance obtained for calibration dispersions, and differences were higher as concentrations increased over 5 mg/L. As mentioned in *Materials and Methods*, if the size of the MWCNT agglomerates is much larger than the wavelength used for absorbance measurements, the former is the main influencing factor affecting the UV-visible absorbance ability of the MWCNTs.[21] The present study's results revealed that different ultrasonic treatments, considering the same concentration of CNTs, could not result in the same absorbance results. This could be attributed to the fact that the size of the agglomerates obtained after ultrasonic treatment was larger than the wavelength used in UV-visible measurements, and for dispersions prepared by sonication bath, CNTs remained more agglomerated than for dispersions prepared by ultrasonic probe. To corroborate this hypothesis, dynamic light scattering analysis was carried out.

6.1.3.3. Dispersion characterization by dynamic light scattering

Dynamic light scattering characterization provided relevant data to analyze and compare the stability of dispersions by the size distributions and rate of agglomeration. The concentrations analyzed corresponded to 30 mg/L, 10 mg/L, 5 mg/L, and 2.5 mg/L for calibration curve dispersions and to 10 mg/L, 5 mg/L, and 2.5 mg/L for verification dispersions. Table 4 shows the Z_{ave} sizes and polydispersity indexes obtained, and the size distribution graphs of the dispersions are included in Figures 6.1.6 to 6.1.9.

Table 6.1.4 Z-average diameter and polydispersity index obtained for multiwalled carbon nanotube dispersions.

	Concentration (mg/L)	CNT-N		CNT-A	
		$Z_{ave,mean}$ (nm)	PdI_{mean} (a.u.)	$Z_{ave,mean}$ (nm)	PdI_{mean} (a.u.)
Calibration dispersions	2.5	255.5	0.383	325.7	0.454
	5	269.5	0.380	348.8	0.447
	10	354.6	0.480	332.7	0.485
	30	297.9	0.477	364.4	0.469
Verification dispersions	2.5	523.2	0.454	672.8	0.516
	5	678.1	0.604	483.3	0.665
	10	830.2	0.655	459.1	0.612

CNT = carbon nanotube; a.u. = arbitrary units; $Z_{ave,mean}$ = mean average Z diameter; PDI = polydispersity index.

As can be seen in Table 4 and Figures 6 to 9, substantial differences for Z_{ave} diameters and polydispersity index existed between calibration and verification dispersions. In the case of the latter, those parameters were quite higher for both types of MWCNTs. Thus, for dispersions prepared by sonication bath, CNTs remained more agglomerated than for dispersions prepared by ultrasonic probe, and the hypothesis established in the previous subsection (*Calibration curves and verification*) was confirmed. The relatively large polydispersity index values indicated that the dispersions were considerably polydisperse, and the Z_{ave} sizes could not have high confidence. This is a well known limitation of the dynamic light scattering technique, but these values were used only for comparative purposes of dispersion quality.

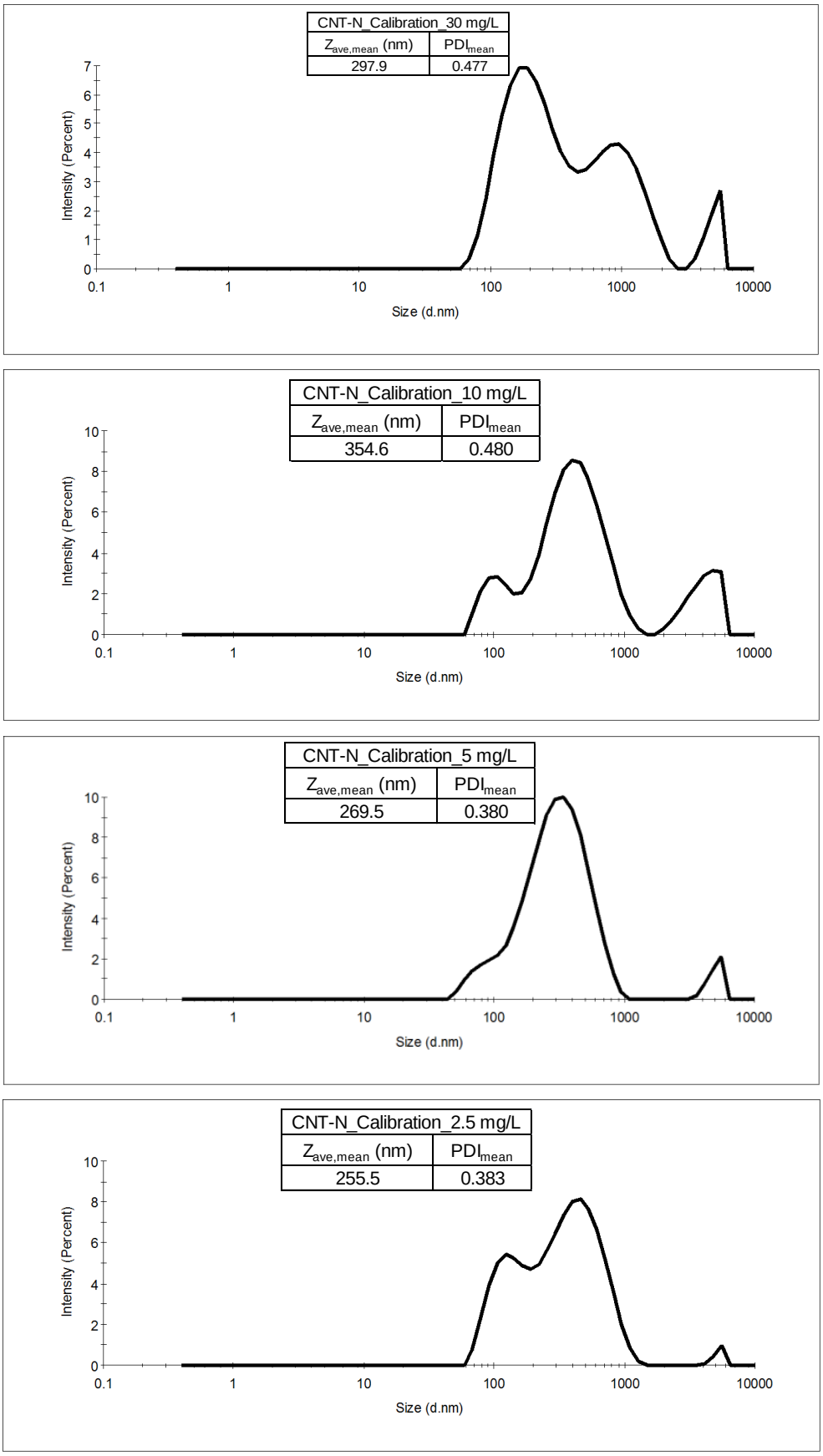

Figure 6.1.6 Size distributions by intensity of CNT-N nanotubes agglomerates in calibration dispersions.

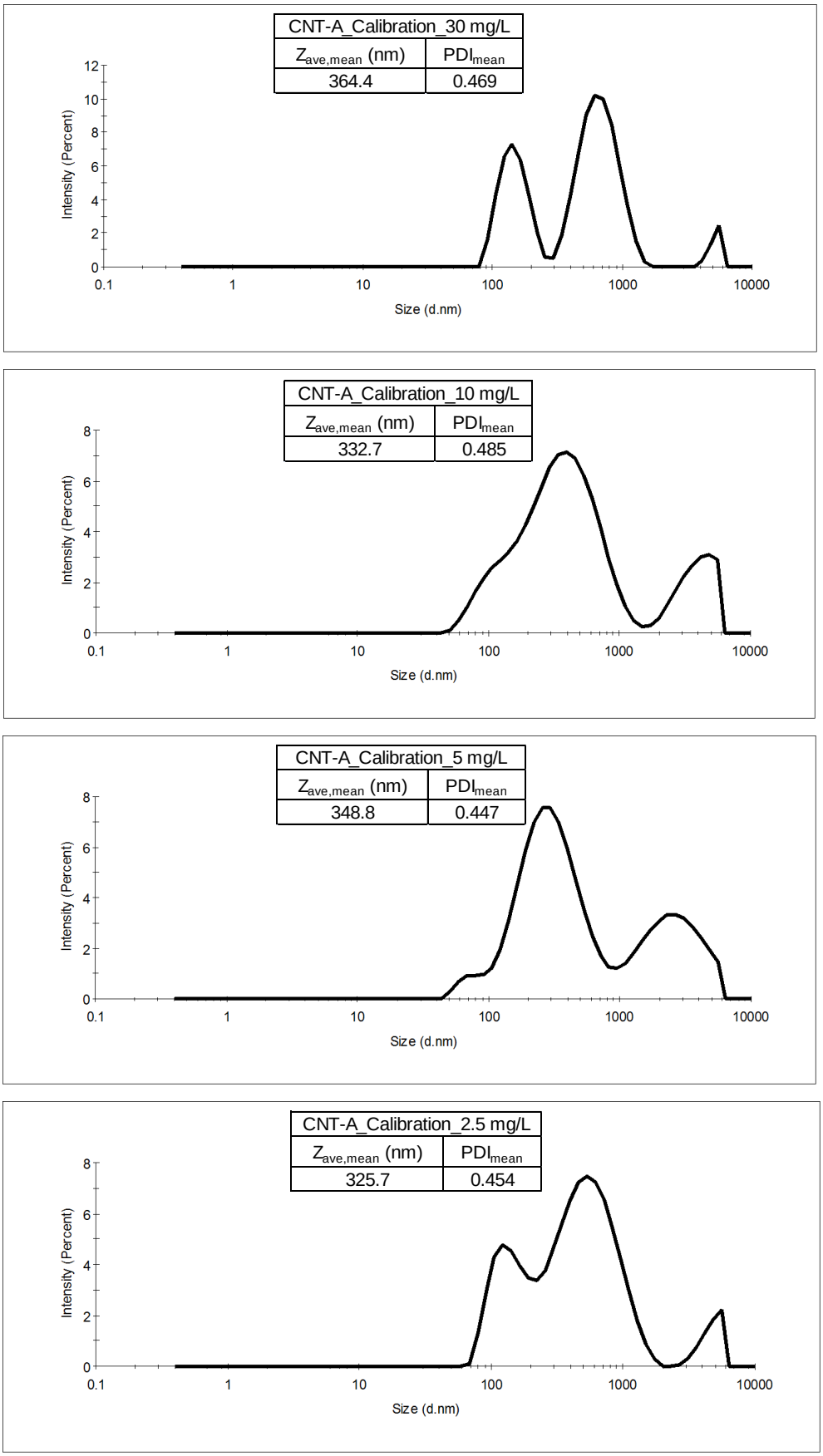

Figure 6.1.7 Size distributions by intensity of CNT-A nanotubes agglomerates in calibration dispersions.

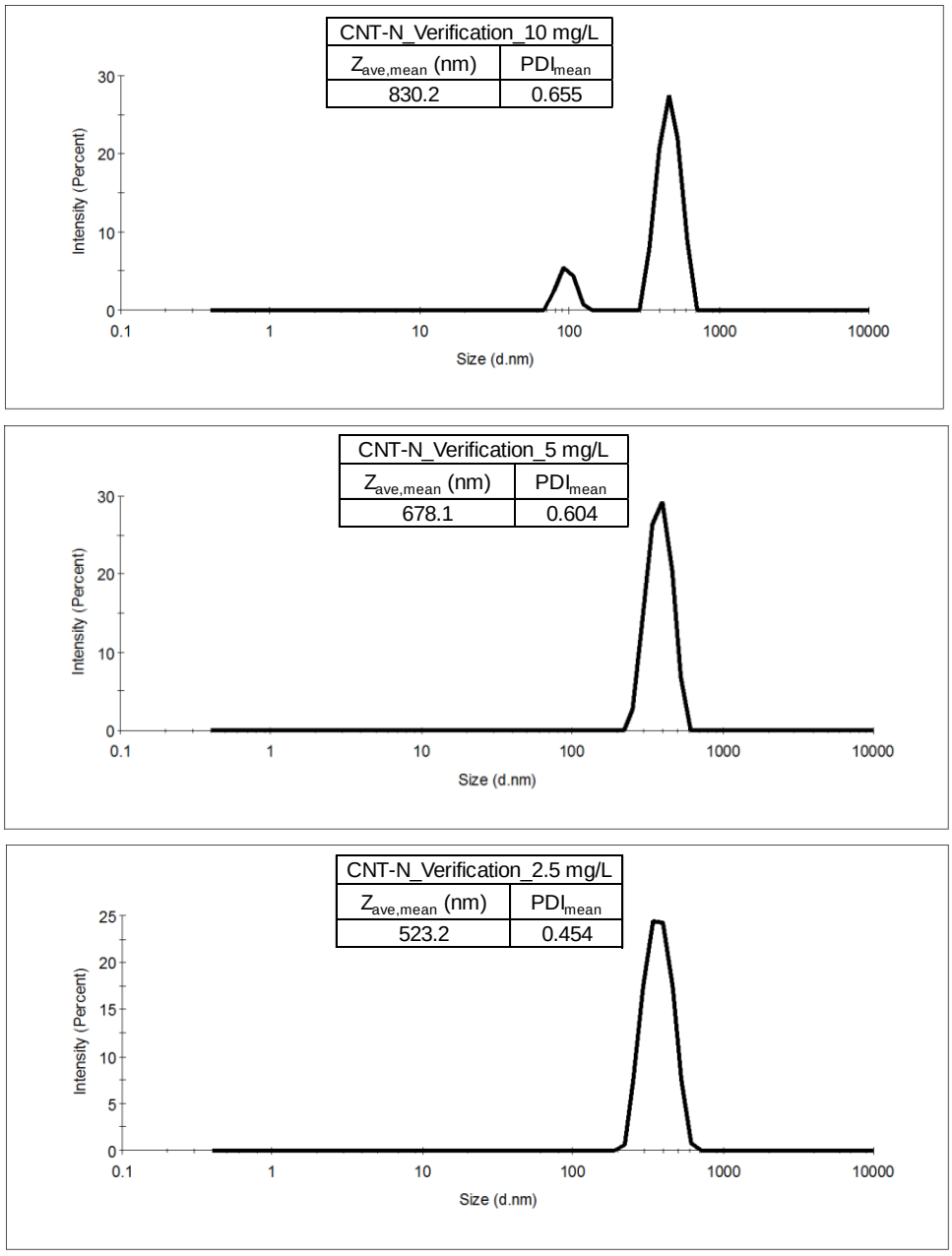

Figure 6.1.8 Size distributions by intensity of CNT-N nanotubes agglomerates in verification dispersions.

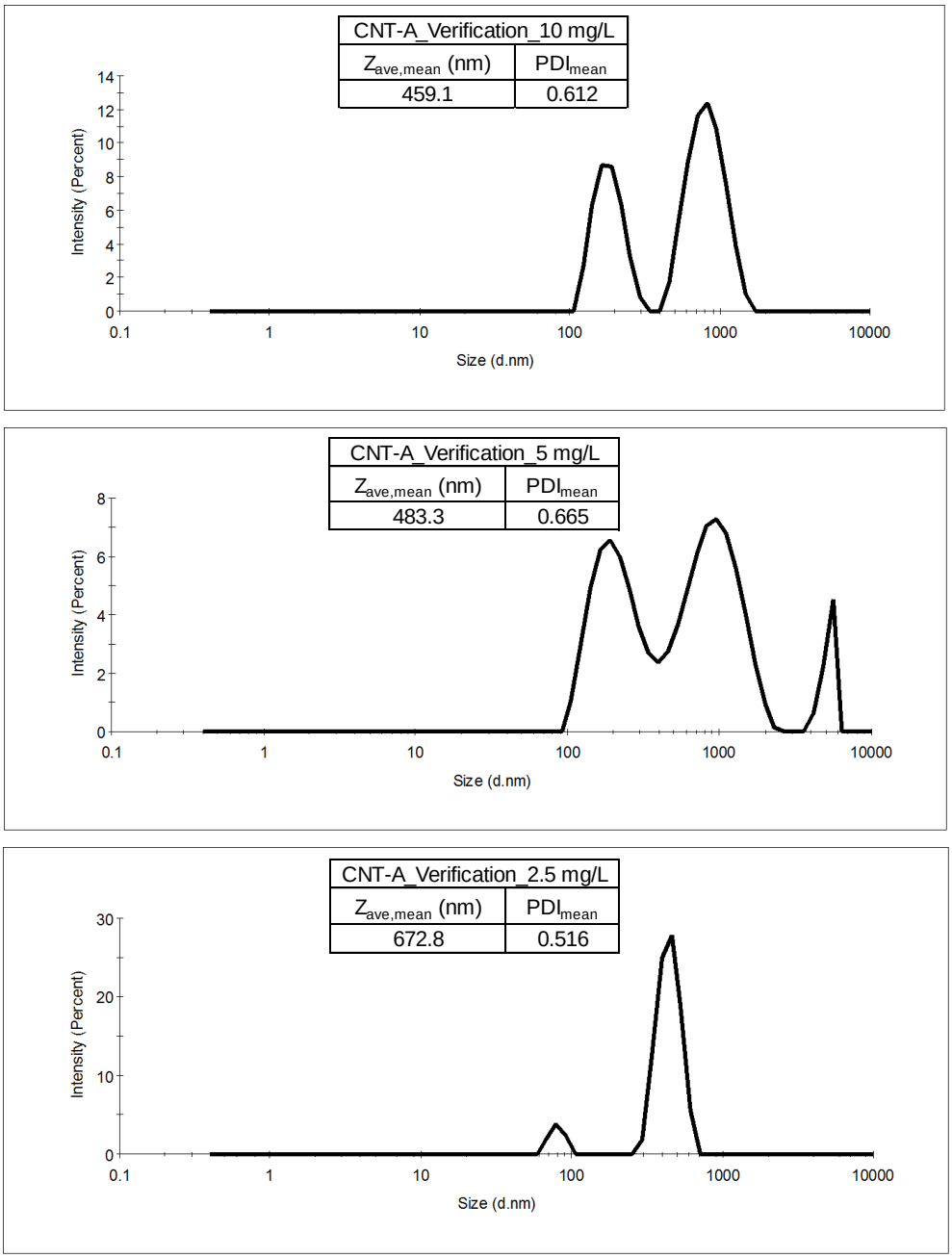

Figure 6.1.9 Size distributions by intensity of CNT-A nanotubes agglomerates in verification dispersions.

Furthermore, dynamic light scattering measurements showed that the sonication parameters selected (time and amplitude) for calibration curve dispersions were appropriate, with Z_{ave} diameters oscillating between 255.5 nm and 354.6 nm for CNT-N and 325.7 nm and 364.4 nm for CNT-A. Polydispersity indexes were in all cases lower than 0.5. Nevertheless, the data obtained for verification dispersions were less acceptable, with Z_{ave} diameters between 523.2 nm and 830.2 nm for CNT-N and 459.1 nm and 672.8 nm for CNT-A. Polydispersity indexes were also higher for these dispersions, exceeding in most cases 0.6. Moreover, differences in Z_{ave} diameters and

polydispersity index for both types of CNTs were observed, as a result of their different physical properties (Table 6.1.1). The concentrations of dispersions analyzed involved also variations in dynamic light scattering parameters measured, with the lowest concentrations producing the most reduced Z_{ave} and polydispersity index.

6.1.3.4. Selection of the dispersion method and ecotoxicity tests

As mentioned in *Introduction*, sonication may modify attributes such as length of nanotubes and, hence, toxicity. Nevertheless, the amount of energy delivered by the sonicators was optimized to avoid damaging and cutting of CNTs, considering a previous study which calculated the minimum energy required to disperse an optimum amount of MWCNTs. On the other hand, the characterization performed suggested that the ultrasonic probe produced lower size distributions and rates of agglomeration than the ultrasonic bath, which demonstrated that the former produced a better optimization of the energy. This occurred despite the fact that the energy delivered by the ultrasonic bath (26.20 kJ) was slightly higher than the energy delivered by the ultrasonic probe (25.35 kJ). Therefore, the sonicator probe was the technique selected to prepare dispersions for ecotoxicity assessment.

With respect to the initial CNT concentrations for these dispersions, it was experimentally observed that the culture medium salt for bacteria (NaCl) reduced their stability. Furthermore, the photometer used to measure the luminescence of bacteria presents limitations for samples with light-absorbent colorants (the case of CNTs) because they can distort the results (the equipment corrects the absorbed light automatically, providing that the absorbances are below 1.800). These drawbacks were overcome by reducing the initial concentrations of MWCNTs to 10 mg/L (and the respective concentration of humic acid to 33.3 mg/L).

The size distribution graphs of the dispersions for ecotoxicity assessment (Figure 6.1.10) indicated a majority of MWCNTs forming larger agglomerates than in the case of dispersions prepared previously for calibration curves and verification. This fact was reasonable considering the differences in the dispersion introduced by the culture medium. Moreover, the agglomerate size was acceptable because previous work on this issue reported similar or even greater Z_{ave} diameters.[36,37] Substantial

[36] Ghosh M, Chakraborty A, Bandyopadhyay M, Mukherjee A. **2011**. Multi-walled carbon nanotubes (MWCNT): Induction of DNA damage in plant and mammalian cells. *J Hazard Mater* 197:327-336.

differences were observed between the size distributions of the CNTs studied, caused by their different physical properties (Table 6.1.1). These divergences were consistent with those observed in the previous subsection because, in the case of 10 mg/L dispersions, Z_{ave} diameters and polydispersity indexes of CNT-N dispersions were higher than those of CNT-A.

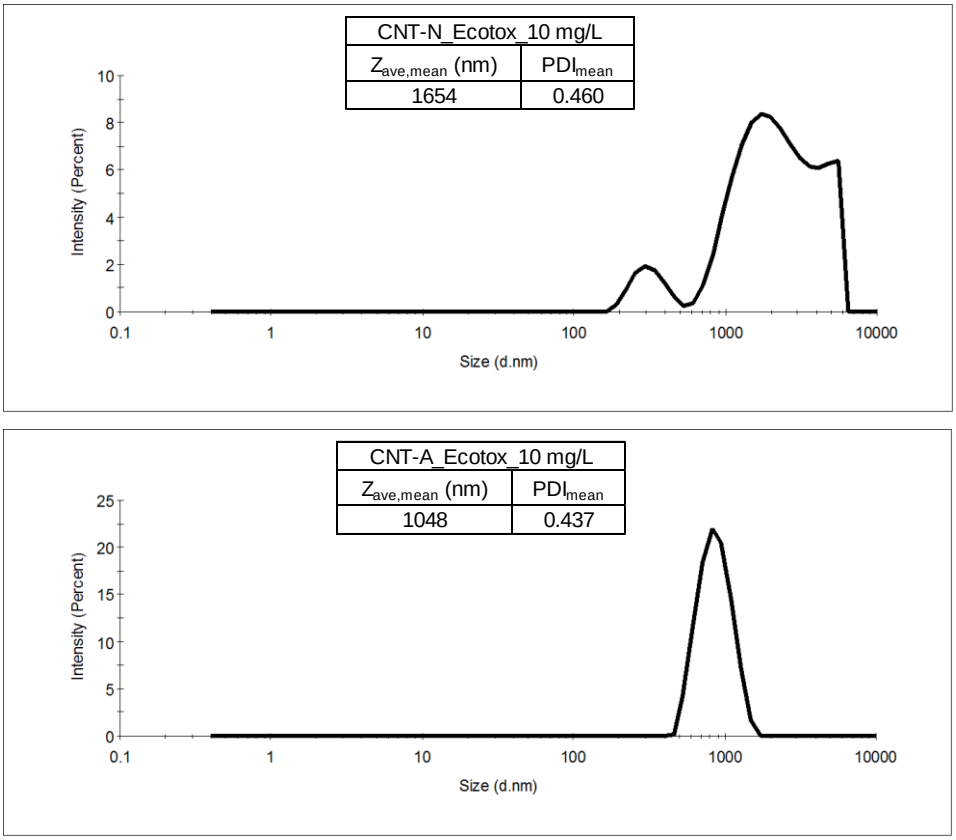

Figure 6.1.10 Size distributions by intensity of MWCNTs dispersions in HA and *Vibrio fischeri* medium for ecotoxicity assessment.

The dispersions for ecotoxicity assessment were also characterized by UV-visible spectroscopy to check whether concentrations of MWCNTs (10 mg/L) produced the expected absorbances according to calibration curve values. The data were slightly higher (3.7% for CNT-N and 2.8 % for CNT-A) than those of calibration curves, probably because of the variations introduced in the sonication process (initial concentrations) and the differences in the agglomerate size. However, if these absorbance values are represented in the calibration curves, the linear fits between the measured absorbances and concentrations remain, with r^2›0.990. Thus, these

[37] Ronzani C, Spiegelhalter C, Vonesch JL, Lebeau L, Pons F. **2012**. Lung deposition and toxicological responses evoked by multi-walled carbon nanotubes dispersed in a synthetic lung surfactant in the mouse. *Arch Toxicol* 86:137-149.

divergences were acceptable and the calibration curves obtained by UV-visible absorbance were useful to measure CNT concentrations in ecotoxicity dispersions.

Independent ecotoxicity tests on *V. fischeri* bacteria were carried out with humic acid to check that the concentrations used were nontoxic and did not alter the toxicity results of CNTs. Values provided in Table 6.1.5 experimentally demonstrated that concentrations of 100 mg/L humic acid did not cause inhibition. Moreover, solutions with higher concentrations (300 mg/L) were tested with the aim of obtaining the EC50 and EC20 endpoints of humic acid.

Table 6.1.5 Toxicity (30-min EC50 and EC20) of humic acid solutions on bacteria *Vibrio fischeri* (mg/L).

Sample	EC50±SD[a]	EC20±SD[a]
HA-100[b]	›50	›50
HA-300[b]	245.0±2.7	165.7±9.4

[a]Standard deviation of tests conducted in triplicate.

[b]Humic acid-100 and Humic acid-300 indicate humic acid concentration (milligrams per liter) of test samples. The dilution series were prepared from these initial concentrations in order to perform the complete test.

EC20, EC50 = toxicant effective concentrations causing 20% and 50% inhibition in light emission, respectively; SD = standard deviation.

Finally, ecotoxicity tests with *V. fischeri* bacteria were conducted. Table 6.1.6 shows the ecotoxicity results obtained for the MWCNTs studied.

Table 6.1.6 Toxicity (30-min EC50 and EC20) of multiwalled carbon nanotube dispersions on bacteria *Vibrio fischeri* (mg/L).

Sample	EC50±SD[a]	EC20±SD[a]
CNT-N/HA	5.4±1.3	1.8±0.6
CNT-A/HA	6.8±2.1	2.0±0.3
HA	›16.7	›16.7
$K_2Cr_2O_7$	19.4±3.5[b]	2.0±0.7[b]

[a]Standard deviation of tests conducted in triplicate.

[b]The validity criteria for the acceptance of the test results were fulfilled since a concentration of 11.3 mg/L $K_2Cr_2O_7$ produced between 20% and 80% inhibition after 30 min of exposure.

CNT = carbon nanotube; EC20, EC50 = toxicant effective concentrations causing 20% and 50% inhibition in light emission, respectively; SD = standard deviation.

The reported data of CNT toxicity on luminescent bacteria are limited to a few studies, as mentioned in *Materials and Methods*;[33,34] and, otherwise, these data are divergent. The literature has demonstrated that SWCNTs are more toxic than MWCNTs to bacteria and microbial communities of aquatic systems.[38,39] However, the EC50 for SWCNTs on *V. fischeri* after an exposure time of 15 min has been reported to be higher than 100 mg/L,[33] although this endpoint ranged between 50 mg/L and 84 mg/L in the case of MWCNTs in another study.[34] Thus, a systematic understanding of their real toxicity is required. Regarding the toxicity results obtained in the present study for MWCNTs, lower values for the EC50 endpoint were observed with respect to the data mentioned.[34] Those divergences are reasonable, given that the physical properties of CNTs studied and the exposure duration in that case (limited to 15 min) were different. In addition, the toxicity of MWCNTs to *V. fischeri* has been reported to be related to the tube size, with the smallest diameters showing greater toxicity.[34,38,40] The influence of size was also observed in the present study because CNT-N nanotubes had lower diameter and length than CNT-A and the concentration required to produce 50% inhibition in light emission of bacteria was smaller than in the case of CNT-A.

The ecotoxicity results depend on the properties of the CNTs and the parameters used in the test, such as exposure time. Hence, standardization of the methodologies to assess their toxic effects is necessary to obtain comparable results between different studies. The present study represents an important contribution to the selection of the most appropriate dispersion methods, which is a key step in the process of standardization. Thus, the ecotoxicity data obtained should be considered in terms of reliability.

6.1.4. Conclusions

The present study has demonstrated that UV-visible absorbance absolutely depends on the sonication method. Therefore, dispersions for calibration curves and for toxicity assessment should be prepared using the same method to achieve the same

[38] Kang S, Herzberg M, Rodrigues DF, Elimelech M. **2008**. Antibacterial effects of carbon nanotubes: Size does matter! *Langmuir* 24:6409-6413.

[39] Kang S, Mauter MS, Elimelech M. **2009**. Microbial cytotoxicity of carbon-based nanomaterials: Implications for river water and wastewater effluent. *Environ Sci Technol* 43:2648-2653.

[40] Chae SR, Therezien M, Budarz JF, Wessel L, Lin S, Xiao Y, Wiesner MR. **2011**. Comparison of the photosensitivity and bacterial toxicity of spherical and tubular fullerenes of variable aggregate size. *J Nanopart Res*13:5121-5127.

absorbance results. Moreover, a new procedure to select the most appropriate measurement wavelength for each type of MWCNT has been proposed. The experimental data obtained in the present study have permitted optimization of the parameters selected to prepare dispersions for conducting an ecotoxicity assessment of MWCNTs on *V. fischeri* bacteria. Considering the lack of standardization in the study of the environmental effects of MWCNTs to date and the few and divergent available data for their toxicity on *V. fischeri*, the reliability of the present study's results should be taken into account.

6.2. Contribution 2

Colloidal stability and ecotoxicity of multiwalled carbon nanotubes: influence of select organic matters

Environmental Toxicology and Chemistry 2015, *Accepted, DOI 10.1002/etc.3172*

Contents

Abstract:

In the last few years, the release of multiwalled carbon nanotubes (MWCNTs) into the environment has raised serious concerns regarding their fate and potential impacts. Aquatic organisms constitute an important pathway for their entrance and transfer throughout the food web, and the current demand for standardization of methodologies to analyze the interactions of MWCNTs with them requires aquatic media that represent natural systems. However, the inherent hydrophobicity of MWCNTs and the substances present in natural waters may greatly affect their stability and bioavailability. The present study analyzes the influence of the most referenced synthetic and natural organic matters (Sigma Aldrich Humic Acid and Suwannee River Natural Organic Matter) in the agglomeration kinetics and ecotoxicity of MWCNTs, with the aim of determining their suitability to fulfill the current standardization requirements. Natural organic matter provides increased colloidal stability to the MWCNTs' dispersions, which results in higher adverse effects on the key invertebrate organism Daphnia magna. *Furthermore, the results obtained with this type of organic matter allow for observation of the important role of the outer diameter and content impurities of MWCNTs in their stability and ecotoxicity on daphnids. Sigma Aldrich humic acid appeared to alter the response of the organisms to carbon nanotubes compared with that observed in the presence of natural organic matter.*

Keywords: Ecotoxicity; Multiwalled carbon nanotubes; Organic matter Dynamic; light scattering; Sonication.

6.2.1. Introduction

Carbon nanotubes (CNTs) exhibit extraordinary physicochemical properties that are useful in many applications in different fields such as chemistry, electronics, energy, materials science, and medicine.[1,2] As a consequence, their large-scale production is increasing, and considerable concerns exist regarding their release into the environment and human exposure.[3-5] The number of industrial facilities for the relatively low-cost production of multiwalled carbon nanotubes (MWCNTs) is experiencing rapid growth compared with that of single-walled carbon nanotubes,[6] and thus a greater release of the former is expected. However, divergent results for the toxicity of MWCNTs have been published,[4,7] and this limited understanding of their environmental, health, and safety aspects poses a threat to their potential applications. These inconsistencies are originated by factors such as impurities, surface modifications, variable structures of carbon nanotubes, and exposure routes.[4]

Aquatic organisms represent one of the most important pathways for the entrance and transfer of MWCNTs throughout the food web in ecosystems.[8,9] Nevertheless, the inherent hydrophobicity of MWCNTs usually results in an agglomeration and settlement behavior, which hinders their stability for ecotoxicological assessment in aqueous systems. In addition, the solution chemistries of aquatic environments influence their stability and thus determine their bioavailability. Previous studies

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[1] Valentini F, Carbone M, Palleschi G. **2013**. Carbon nanostructured materials for applications in nano-medicine, cultural heritage, and electrochemical biosensors. *Anal Bioanal Chem* 405:451-465.

[2] Heister E, Brunner EW, Dieckmann GR, Jurewicz I, Dalton AB. **2013**. Are carbon nanotubes a natural solution? Applications in biology and medicine. *ACS Appl Mater Interfaces* 5:1870-1891.

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[4] Liu Y, Zhao Y, Sun B, Chen C. **2013**. Understanding the toxicity of carbon nanotubes. *Accounts Chem Res* 46:702-713.

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[8] Petersen EJ, Akkanen J, Kukkonen JVK, Weber WJ. **2009**. Biological uptake and depuration of carbon nanotubes by Daphnia magna. *Environ Sci Technol* 43:2969-2975.

[9] Yu ZG, Wang WX. **2013**. Influences of ambient carbon nanotubes on toxic metals accumulation in Daphnia magna. *Water Res* 47:4179-4187.

have analyzed the interactions between MWCNTs and the substances present in natural waters, such as monovalent and divalent salts as well as natural organic matter (NOM).[10-12] High ionic strength and low pH induce the colloidal destabilization of MWCNTs, whereas humic substances (the major fraction in NOM) promote their stabilization.[13,14] The adsorption of NOM by MWCNTs also has been studied for separation and purification applications for drinking water.[15,16]

Humic acid and fulvic acid are the components of humic substances distributed in aquatic environments.[17] Humic acid is the main fraction of NOM and exhibits a higher molecular weight than fulvic acid.[18] It has been widely used in ecotoxicity assessments of MWCNTs, especially in its commercially available form synthesized by Sigma-Aldrich.[12,19,20] Furthermore, Suwannee River NOM and humic acid (SR-NOM and SR-humic acid, respectively)[17] are the most extensively used natural organic substances to study the bioavailability of MWCNTs.[10,11,14,21,22] Their key advantage is the simulation of the real ecosystems in the toxicity assays, unlike laboratory-

[10] Saleh NB, Pfefferle LD, Elimelech M. **2008**. Aggregation kinetics of multiwalled carbon nanotubes in aquatic systems: Measurements and environmental implications. *Environ Sci Technol* 42:7963-7969.

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[15] Lu C, Su F. **2007**. Adsorption of natural organic matter by carbon nanotubes. *Sep Purif Technol* 58:113-121.

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[21] Hyung H, Fortner JD, Hughes JB, Kim JH. **2007**. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environ Sci Technol* 41:179-184.

[22] Chae SR, Xiao Y, Lin S, Noeiaghaei T, Kim JO, Wiesner MR. **2012**. Effects of humic acid and electrolytes on photocatalytic reactivity and transport of carbon nanoparticle aggregates in water. *Water Res* 46:4053-4062.

synthesized humic substances. Natural organic matter is a more representative sample than natural humic acid of what is found naturally, composed of chemically complex polyelectrolytes with varying molecular weights, and produced mainly from the decomposition of plant and animal residues.[11]

The type of organic matter used in the tests has been shown to influence the adverse effects of MWCNTs on aquatic organisms.[23,24] Previous studies have compared the behavior of MWCNTs in the presence of organic matter from different sources, namely, Sigma-Aldrich Humic Acid and soil loam,[12] NOM and humic substances,[11,16,24] and different humic acids.[13,25-27] However, the understanding of the behavior of MWCNTs in the aquatic environment needs operational procedures that represent natural systems,[28] and the current demand for standardization of materials and methods to analyze their ecotoxicity remains unsolved.[29]

The present study compares the agglomeration kinetics and ecotoxicity of MWCNTs in the presence of the most referenced synthetic and natural organic matters (Sigma Aldrich humic acid and SR-NOM, respectively), with the aim of determining which is most appropriate to fulfill the current regulation requirements. The standardization approach of the present study also includes the calculation and optimization of the energy delivered to the MWCNTs during the preparation of dispersions. Some previous works did not consider this aspect,[8,24] resulting in significant damage to the MWCNTs, which may alter their behavior within the context of toxicological

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[23] Kim KT, Edgington AJ, Klaine SJ, Cho JW, Kim SD. **2009**. Influence of multiwalled carbon nanotubes dispersed in natural organic matter on speciation and bioavailability of copper. *Environ Sci Technol* 43:8979-8984.

[24] Edgington AJ, Roberts AP, Taylor LM, Alloy MM, Reppert J, Rao AM, Mao J, Klaine SJ. **2010**. The influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. *Environ Toxicol Chem* 29:2511–2518.

[25] Zhou X, Shu L, Zhao H, Guo X, Wang X, Tao S, Xing B. **2012**. Suspending multi-walled carbon nanotubes by humic acids from a peat soil. *Environ Sci Technol* 46:3891-3897.

[26] Tian X, Li T, Yang K, Xu Y, Lu H, Lin D. **2012**. Effect of humic acids on physicochemical property and Cd(II) sorption of multiwalled carbon nanotubes. *Chemosphere* 89:1316-1322.

[27] Wang X, Shu L, Wang Y, Xu B, Bai Y, Tao S, Xing B. **2011**. Sorption of peat humic acids to multi-walled carbon nanotubes. *Environ Sci Technol* 45:9276-9283.

[28] Park S, Woodhall J, Ma G, Veinot JGC, Cresser Boxall ABA. **2014**. Regulatory ecotoxicity testing of engineered nanoparticles: Are the results relevant to the natural environment? *Nanotoxicology* 8:583-592.

[29] Savolainen K, Backman U, Brouwer D, Fadeel B, Fernandes T, Kuhlbusch T, Landsiedel R, Lynch I, Pylkk€anen L. Nanosafety in Europe 2015-2025: Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations. EDITA, Helsinki, Finland. 2013.

testing.[4,30] Inhibitory effects on the key invertebrate organisms for regulatory testing *Daphnia magna* were studied, considering also that several experimental data on toxicity of MWCNTs toward them have been published.[8,24,31-33]

6.2.2. Materials and Methods

6.2.2.1. Materials for dispersions

Multiwalled carbon nanotubes were obtained from commercial sources and from the Joint Research Centre-European Commission Repository, who provided their physical descriptions and impurity percentages (Table 6.2.1). All of them were produced via catalytic chemical vapor deposition. Additional purification steps were not performed with MWCNTs to analyze the influence of the amounts of impurities on their toxic effects.

Table 6.2.1 Suppliers, physical descriptions and impurity percentages of the multiwalled carbon nanotubes studied.

Code	Supplier identification	Outer diameter (nm)	Length (nm)	Impurities (remaining catalyst metals and amorphous carbon) (%)
CNT-1	Nanocyl NC7000	6-24	2000-5000	‹5
CNT-2	Arkema Graphistrength C100	10-15	1000-10000	‹10
CNT-3	JRCNM04000a	9-18	400-1300	16.2
CNT-4	JRCNM04001a	28-99	1600-6500	18.1

CNT = carbon nanotube.

Standard Suwannee River NOM obtained from the International Humic Substances Society was used as a model NOM. Humic acid, selected as a model synthetic organic matter, was purchased from Sigma-Aldrich. Both substances were used without any further purification.

[30] Taurozzi JS, Hackley VA, Wiesner MR. **2011**. Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment issues and recommendations. *Nanotoxicology* 5:711-729.

[31] Petersen EJ, Pinto RA, Mai DJ, Landrum PF, Weber WJ Jr. **2011**. Influence of polyethyleneimine graftings of multi-walled carbon nanotubes on their accumulation and elimination by and toxicity to Daphnia magna. *Environ Sci Technol* 45:1133-1138.

[32] Arndt DA, Chen J, Moua M, Klaper RD. **2014**. Multigeneration impacts on Daphnia magna of carbon nanomaterials with differing core structures and functionalizations. *Environ Toxicol Chem* 33:541-547.

[33] ZhuX, ZhuL,ChenY,Tian S. **2008**. Acute toxicities of sixmanufactured nanomaterial suspensions to Daphnia magna. *J Nanopart Res* 11:67-75.

All stock solutions and dispersions were prepared in ultrapure water, produced by a Milli-Q water filtration system (Millipore). The rest of chemicals used were p.a. grade and obtained from Sigma-Aldrich and Scharlab.

6.2.2.2. Preparation of MWCNT dispersions

To prepare the organic matter solutions, 20 mg humic acid/SR-NOM were added to 1 L of either culture medium of the organisms or ultrapure water alone, and mixed on a magnetic stirrer for 72 h at 20±2 °C. This time was sufficient to achieve a complete dissolution of the organic matter. Hence, a subsequent step of centrifugation or filtration to extract the supernatants was not necessary. Humic acid and SR-NOM concentrations were the same, to obtain comparable results for the agglomeration kinetics and ecotoxicity of MWCNTs. They were selected by taking into account the amounts in natural waters.[20,34,35]

The dispersions were obtained by adding 10 mL of humic acid/SR-NOM solution to 0.5 mg MWCNTs in 20-mL glass scintillation vials and then sonicated with an ultrasonic homogenizer, a widely accepted method that ensures reasonable stability.[13,24] The MWCNT concentrations were selected according to the short-term endpoints reported in the literature for D. *magna* tests.[3,31,33] The ultrasonic homogenizer (VIBRACELL-VCX750, SONICS&MATERIALS) operated with a standard probe (136-mm length and 13-mm diameter), at a frequency of 20 kHz, continuous mode for 16 min and output power fixed at 750 W at 40% amplitude. The calorimetric method described by Taurozzi et al.[30] was used to calculate the sonication time and amplitude required to optimize the acoustic energy delivered by the probe. Considering the previously reported energy required to achieve the maximum degree of dispersion of MWCNTs in aqueous solution without damaging CNTs,[36] we established that the total amount of energy supplied to the dispersions should not exceed 30 KJ.

A 600 mL borosilicate glass beaker was filled with 500 mL thermally equilibrated MilliQ water, in order to calculate the energy delivered by the sonication method. Its

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[34] Bennett SW, Adeleye A, Ji Z, Keller AA. **2013**. Stability, metal leaching, photoactivity and toxicity in freshwater systems of commercial single wall carbon nanotubes. *Water Res* 47:4074-4085.

[35] Cupi D, Hartmann NB, Baun A. **2015**. The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide, and titanium dioxide nanoparticles with Daphnia magna. *Environ Toxicol Chem* 34:497-506.

[36] Yu J, Grossiord N, Koning CE, Loos J. **2007**. Controlling the dispersion of multi-wall carbon nanotubes in aqueous surfactant solution. *Carbon* 45:618-623.

temperature and mass were measured with an uncertainty of ±0.1 °C and ±0.1 g, respectively. The beaker was placed in the sonicator chamber and the tip was immersed to a position 2.5 cm below the liquid surface. The temperature probe was mounted (using a clamp) at 2.5 cm depth and 1 cm away from the sonicator probe. The sonicator output selected was 40% amplitude (considering previous dispersion tests carried out in our laboratory), operating in continuous mode. The temperature increase of the water was recorded for 6.5 minutes with a time resolution of 30 seconds.

Calculation of the delivered acoustic energy was performed obtaining the best linear fit (R^2›0.990) between the measured temperature and time using least squares regression. The effective delivered power was determined using the Equation 6.2.1:

$$P = \frac{\mathrm{d}T}{\mathrm{d}t} M C_p \qquad (6.2.1)$$

where P is the delivered acoustic power (W), dT/dt is the slope of the regression curve, M is the mass of liquid (g), and C_p is the specific heat of the liquid ($J \cdot g^{-1} \cdot °C^{-1}$).

The effective delivered acoustic power (P) was 25.985 W. The linear fits between the measured temperature as function of time using least squares regression are represented in Figure 6.2.1.

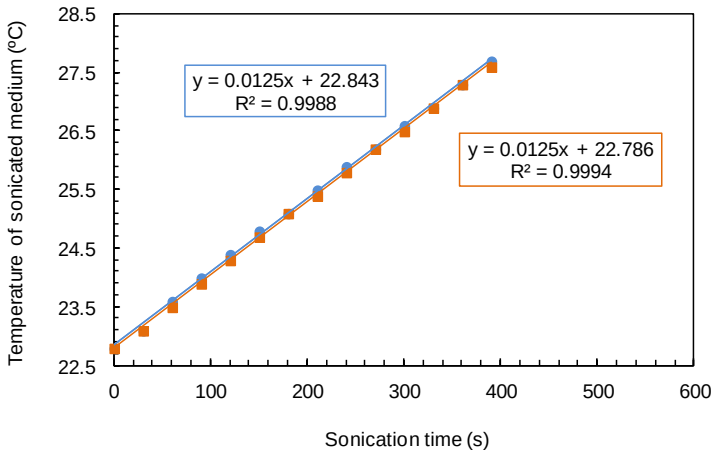

Figure 6.2.1 Linear fits between the measured temperature as function of time sonicator probe.

The total amount of energy delivered (Equation 6.2.2) was obtained considering the applied power and also the total amount of time that the water is subjected to the ultrasonic treatment:

$$E = P \times t \qquad (6.2.2)$$

where *E* is the total amount of energy (J), *P* is the delivered acoustic power (W) and *t* is the total amount of time (s).

Considering the sonication time selected (16 min), the total amount of energy delivered (E) was 24946 J for sonicator probe. These values are in accordance with the maximum specified (30 KJ), to avoid damaging and cutting of CNTs. It is important to consider that the actual volumes and temperatures of MWCNTs dispersions were different from that used in the calculation of the energy delivered by the sonication methods. However, this aspect is noted in the calorimetric method,[30] which is simply intended to allow the reporting and transference of sonication power levels between users, not to measure the actual fraction of power utilized for powder disruption under specific dispersion conditions.

During sonication, the vials were held in an ice bath to minimize temperature rising of the sample, and the probe was inserted between the upper quarter and upper half of the dispersion volume in the vials. These conditions were essential to maximize the liquid-probe surface area exposed to the acoustic waves, as well as the container wall surface to volume ratio for dissipation of heat by the cooling bath.[30]

The dispersions were characterized and tested immediately after their preparation. Subsequent steps of settling or centrifugation were avoided, because potential changes such as agglomeration and sedimentation were considered reactions occurring in the test systems.

6.2.2.3. Characterization of MWCNT dispersions

Dynamic light scattering, ultraviolet-visible (UV/Vis) spectroscopy, and scanning electron microscopy (SEM) characterization were conducted in all the dispersions immediately after their preparation and at the end of the ecotoxicity tests. A slight shaking for homogenization preceded the characterization of the dispersions at the end of the tests. Sampling the aquatic phase would have required additional settling or centrifugation steps, because sedimented or agglomerated MWCNTs were not clearly observed.

The stability of the dispersions was assessed by measuring the variation in scattered light intensity and calculated average zeta-sizes as a function of time. For this purpose, Zeta-average diameter (Z_{ave}) and polydispersity index values were obtained

by dynamic light scattering measurements in a Malvern Zetasizer Nano ZS instrument, considering the data generated from 10 repeated measurements.

Ultraviolet-visible spectroscopy was used to determine quantitatively MWCNT concentration in dispersions and to study the influence of humic acid and SR-NOM in the agglomeration kinetics of MWCNTs. This is one of the most reported techniques in the last ten years to determine concentrations, given its rapidness and low cost.[37,38] The absorbances of dispersions with previously known concentrations were measured to obtain the corresponding calibration curves. These calibration dispersions were prepared with humic acid/SR-NOM dissolved in ultrapure water (without adding culture medium), with starting concentrations of 50 mg/L MWCNTs. Dilution levels, as well as the UV/Vis absorbance results of the calibration dispersions, can be found in *Results and Discussion* section. The apparent concentrations of MWCNTs in batch dispersions (prepared with humic acid/SR-NOM dissolved in culture medium) were obtained from the UV/Vis absorbances of the calibration dispersions. All of the measurements were conducted immediately after sonication processes at 530 nm, using an UV/Vis/NIR (ultraviolet-visible-near infrared) spectrophotometer (Lambda 950, PerkinElmer) and quartz cells with 10-mm path length. The selection of the wavelength was carried out considering that previously reported for MWCNTs.[39] Although the absorbance peaks of the nanotubes studied were observed at lower wavelengths, saturation of the spectrophotometer was reached in this region of the spectrum. Moreover, the absorbance peaks of organic matter occur at lower wavelengths[21,40] and might have interfered with those of CNTs. The absorbance values of humic acid and SR-NOM were negligible at 530 nm and did not alter those obtained for MWCNTs. Nonetheless, the measurements were carried out considering humic acid and SR-NOM as background substances and subtracting their absorbance by the "autozero" function of the spectrophotometer. The absorbance of the nutrients in the culture medium was also

[37] Li ZF, Luo GH, Zhou WP, Wei F, Xiang R, Liu YP. **2006**. The quantitative characterization of the concentration and dispersion of multi-walled carbon nanotubes in suspension by spectrophotometry. *Nanotechnology* 17:3692-3698.

[38] Khripin CY, Tu X, Howarter J, Fagan J, Zheng M. **2012**. Concentration measurement of length-fractionated colloidal single-wall carbon nanotubes. *Anal Chem* 84:8733-8739.

[39] Marsh DH, Rance GA, Zaka MH, Whitby RJ, Khlobystov AN. **2007**. Comparison of the stability of multiwalled carbon nanotube dispersions in water. *Phys Chem Chem Phys* 9:5490-5496.

[40] Pokhrel LR, Dubey B, Scheuerman PR. **2013**. Impacts of select organic ligands on the colloidal stability, dissolution dynamics, and toxicity of silver nanoparticles. *Environ Sci Technol* 47:12877-12885.

subtracted for batch dispersion measurements, although their absorbance spectrum between 400 nm and 1200 nm was observed to be negligible.

Furthermore, SEM imaging was performed to support the results obtained by the previous characterization, using a Zeiss apparatus (ULTRA PLUS model). A drying process (24 h under ambient temperature) prepared the SEM samples. During this period, MWCNTs possibly formed larger agglomerates, and organic matter and culture media substances may have crystallized. Thus, this ultimate disposition was not totally comparable with what happened when they were in dispersion but showed the appearance of nanotubes after sonication and ecotoxicity tests.

6.2.2.4. *D. magna* acute immobilization tests

Neonates of *D. magna* used (aged less than 24 h) were obtained from Microbiotests. The assays were performed following the prescriptions of the Organisation for Economic Co-operation and Development "*Daphnia* sp., Acute Immobilization Test" (Guideline 202).[41]

The tubes containing dormant eggs (ephippia) of the organisms were stored in a refrigerator at 5±2 °C. The hatching of the daphnids was carried out by transferring the ephippia into 30 ml culture medium (composition detailed below), 72 h prior to the start of the toxicity tests. They were maintained at 20±2 °C under continuous illumination of a minimum of 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ (light intensity at the top of the cultures, measured in the wavelength range of 400-700 nm). The largest hatching occurred between 72 h and 80 h of incubation and the organisms were collected at the latest 90 h after the start of the incubation. Prior to the test, a 2h pre-feeding was applied with a suspension of *Spirulina* microalgae. This food uptake provided neonates with an energetic reserve and precluded mortality by starvation (which would bias the test results), since the organisms were not fed during the subsequent test. Culture medium was used to prepare HA/SR-NOM solutions for MWCNTs dispersions. The organisms were exposed to five dilutions of the test substances over a period of 48 hours in multiwell test plates. For this purpose, each well was filled with 10 mL of the respective concentrations, in the sequence of increasing toxicant dilutions and five neonates were transferred into each well with a micropipette. The controls and each test concentration were assayed in four replicates (with 5 neonates each well) for a statistically acceptable evaluation of the effects. A Parafilm strip (plastic paraffin

[41] Daphnia sp. Acute Immobilisation Test. OECD Guideline 202. Organization for Economic Cooperation and Development (OECD). Paris, France. Adopted 13 April 2004.

film) was put on the plates, and they were covered tightly. Incubation was performed at 20±2 °C in darkness. After 24h and 48h incubation, the multiwell plates were positioned on the stage of a light table and the number of dead and immobilized neonates in each well was recorded. The neonates which were not able to swim after gentle agitation of the liquid for 15 seconds were considered to be immobilized, even if they could still move their antennae.

The culture and dilution medium for *D. magna* was prepared by adding 25 mL of the stock solutions 1-4 (they were stored in the dark at 4 °C) to 1 L ultrapure water.

-Stock solution 1: 11.76 g $CaCl_2 \cdot 2H_2O$ in 1 L ultrapure water

-Stock solution 2: 4.93 g $MgSO_4 \cdot 7H_2O$ in 1 L ultrapure water

-Stock solution 3: 2.59 g $NaHCO_3$ in 1 L ultrapure water

-Stock solution 4: 0.23 g KCl in 1 L ultrapure water

Before use, the solution was equilibrated by bubbling with air for at least 15 minutes. The dissolved oxygen concentration was around 7 mg/L. After equilibration, the pH was adjusted to 8.2-8.3, with either 1 M HCl or 1 M NaOH.

Each test involved 5 concentrations starting at 50 mg/L, and each concentration used 20 neonates (distributed in 4 replicates). The total number of dead and immobile neonates during exposure for 48 h was calculated for each dilution, and the concentrations bringing 20% and 50% immobilization (EC20 and EC50, respectively) as well as their associated 95% confidence limits were determined by regression analysis in Excel 2007 (Microsoft).

Because an essential aim of the present study was the assessment of the influence of the type of organic matter on the ecotoxicity of MWCNTs, the concentration of nutrients and pHs of the media were adjusted to the specific requirements for the test organisms. The initial pH values of culture medium and test dispersions were adjusted to 8.2 to 8.3, as high as possible considering the previously mentioned fact that low pH induces the colloidal destabilization of CNTs.[13] To check the validity of the test procedures, additional tests were carried out with the reference chemical potassium dichromate ($K_2Cr_2O_7$). The SR-NOM and humic acid solutions were also independently analyzed to verify that the concentrations used did not induce toxic responses.

6.2.3. Results and Discussion

6.2.3.1. Characterization of MWCNT dispersions

Characterization by dynamic light scattering

A previous characterization by dynamic light scattering was performed with the 50 mg/L MWCNT calibration dispersions (Table 6.2.2 and Figures 6.2.2 and 6.2.3).

Table 6.2.2 Z-average diameters (Z_{ave}) and polydispersity indexes (PDI) of CNTs agglomerates in calibration dispersions.

	CNT-1		CNT-2		CNT-3		CNT-4	
	SR-NOM	HA	SR-NOM	HA	SR-NOM	HA	SR-NOM	HA
$Z_{ave,mean}$ (nm)	253.7	251.8	199.1	208.1	180.2	257.8	601.1	587.4
PDI_{mean}	0.405	0.572	0.426	0.415	0.499	0.436	0.280	0.394

CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

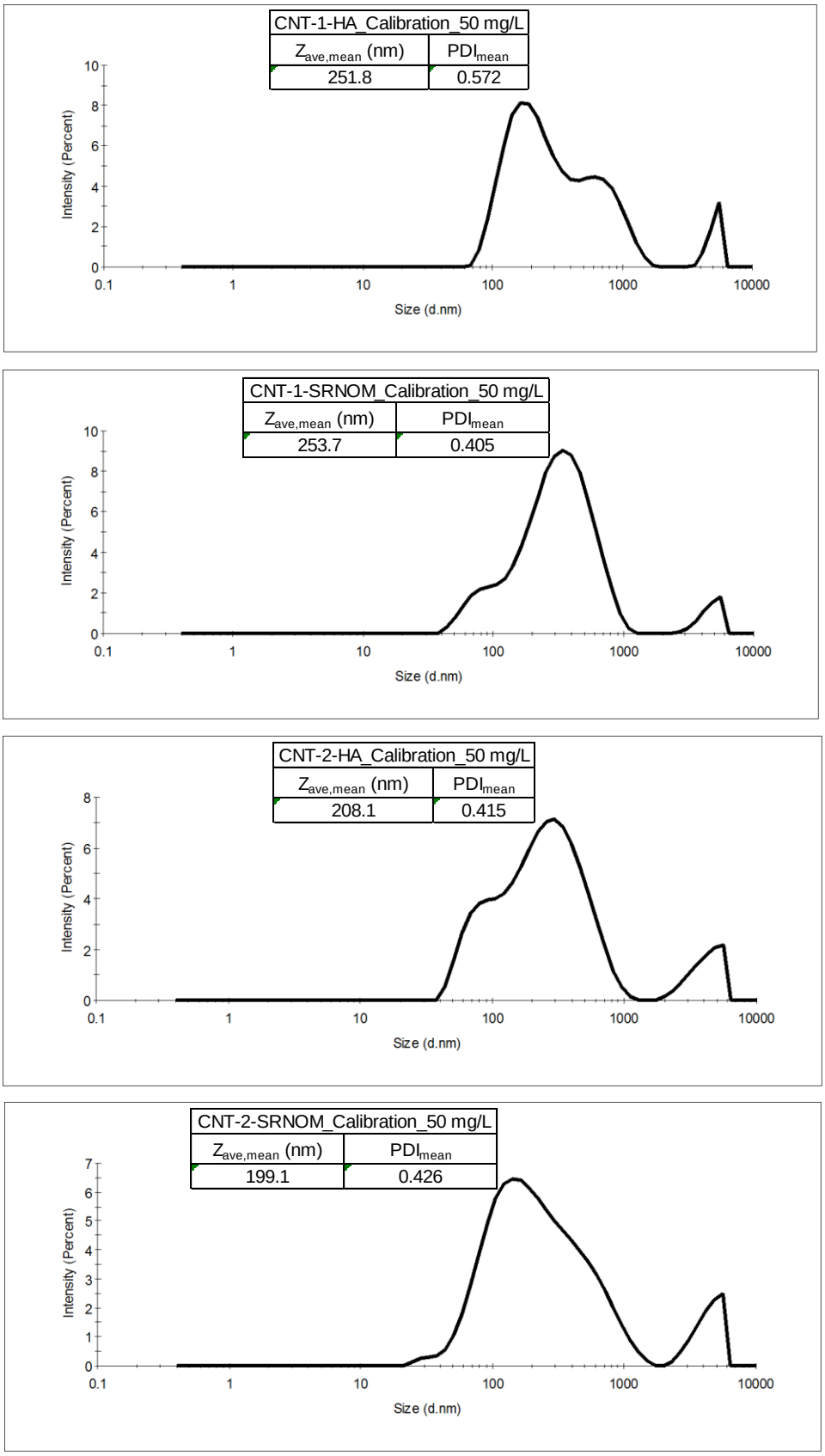

Figure 6.2.2 Size distributions by intensity of CNT-1 and CNT-2 agglomerates in calibration dispersions.

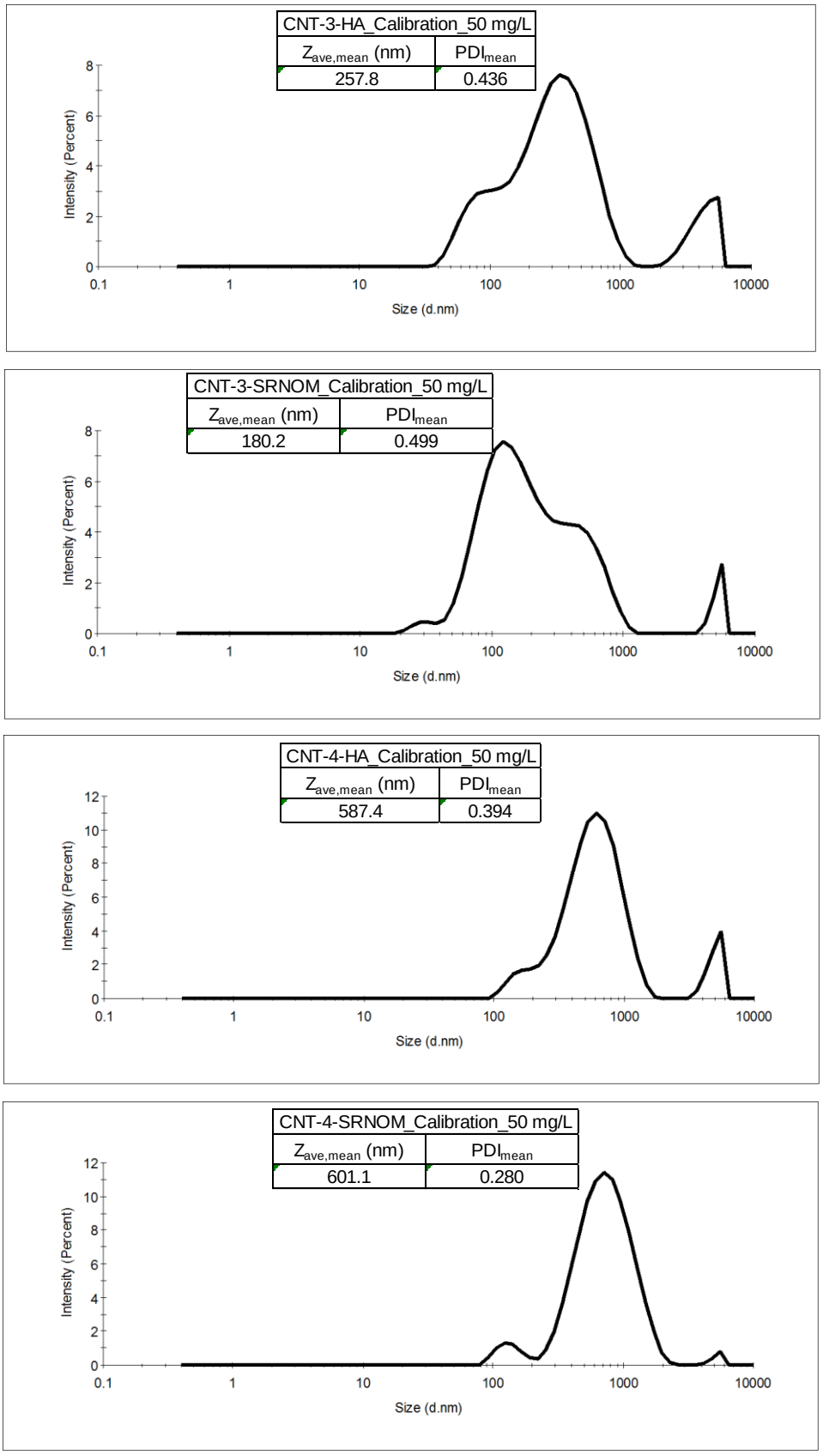

Figure 6.2.3 Size distributions by intensity of CNT-3 and CNT-4 agglomerates in calibration dispersions.

With respect to the initial MWCNT concentrations for these dispersions, the addition of culture medium for the toxicity tests substantially increased the parameters measured with dynamic light scattering. Table 6.2.3 shows Z_{ave} and polydispersity index of dispersions prepared for ecotoxicity assessment, at the beginning and end of the tests.

Table 6.2.3 Z-average diameters (Z_{ave}) and polydispersity indexes (PDI) of CNTs agglomerates in batch dispersions at the beginning and end of the tests.

		CNT-1		CNT-2		CNT-3		CNT-4	
		SR-NOM	HA	SR-NOM	HA	SR-NOM	HA	SR-NOM	HA
$Z_{ave,mean}$ (nm)	0 h	1384	1821	1760	1914	2272	1284	2034	2249
	48 h	3187	2805	1763	1845	2822	1639	1719	2073
PDI_{mean}	0 h	0.809	0.753	0.805	0.725	0.773	0.708	0.533	0.418
	48 h	1.000	1.000	0.822	0.731	0.835	0.813	0.483	0.382

CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

Except for the results obtained for CNT-4, polydispersity index values were higher than 0.7. The calibration dispersions showed quite different polydispersity index values in all cases, and lower than 0.5 for most. Similarly, although the batch dispersions presented micrometric agglomerates, the Z_{ave} diameters obtained for the calibration dispersions were the lowest, ranging from 200 nm to 600 nm. The considerable Z_{ave} and polydispersity index values obtained for the batch dispersions pose certain limitations of the dynamic light scattering technique for the characterization of the nanotube agglomerates. Nevertheless, it can be used as a tool to compare the relative nanotube stability and agglomeration.[12,42] Dynamic light scattering results were useful to analyze the differences between the dispersions prepared with the 2 types of organic matter studied, and in any case SEM images and UV/Vis measurements provided additional data for characterization. Conversely, the size of agglomerates and polydispersity index values in the batch dispersions could be reduced by decreasing the initial concentration of MWCNTs. Previous studies have reported that high concentrations in the dispersion process result in an increased nanoparticle collision frequency and also may induce agglomerate or aggregate formation as particles collide and coalesce.[30,42] However, sufficiently high

[42] Kennedy AJ, Gunter JC, Chappell MA, Goss JD, Hull MS, Kirgan RA, Steevens JA. **2009**. Influence of nanotube preparation in aquatic bioassays. *Environ Toxicol Chem* 28:1930-1938.

initial concentrations, which led to inhibitory effects, were necessary to assess the ecotoxicity of the dispersions. Considering the previously reported average nanotube Z_{ave} sizes for ecotoxicity tests with D. magna,[8,24] the results obtained in the present study were generally higher. As mentioned, these previous works did not consider the acoustic energy supplied by the ultrasonic probe during the preparation of dispersions. Significant damage to the MWCNTs was observed in both cases, even forming a high fraction of functional groups,[8] which may alter their ecotoxicity. Average diameters ranging from 800 nm to more than 2 mm have been reported,[33] more similar to those obtained in the present study. Furthermore, the settling of the dispersions after sonication to test only the supernatant is a common procedure to discard any undispersed MWCNTs and obtain better Z_{ave} and polydispersity index values,[23,24] but it does not represent what takes place in natural environments. Agglomerated and sedimented nanotubes are expected to be very persistent.[14] Moreover, aquatic environments are not static media, and agglomerates also might be present in suspension in lakes and rivers. Thus, the approach in the present study constituted a better approximation to a realistic situation.

The type of organic matter used seemed to be related to variations in the stability of the dispersions. At the beginning of the tests, three of the MWCNTs analyzed showed lower Z_{ave} for SR-NOM dispersions. After 48 h of exposure, a clear trend was not observed in the agglomerate sizes, neither in the presence of SR-NOM nor in the presence of humic acid. However, in the case of the dispersions prepared for calibration curves without culture medium, SR-NOM produced a decrease in Z_{ave} or polydispersity index values for all of the nanotubes studied, suggesting that it provided an increased stability to the dispersions with respect to humic acid. The present study performed an evaluation of the dispersant capability of two types of organic matter for a single culture medium. The nutrient salts of the medium were a key aspect of MWCNTs stability; thus, their behavior in ultrapure water should be taken as a reference to achieve the harmonization of the methodologies.

Regarding the size distributions of the four MWCNTs studied, differences were observed between them, probably because of their different physical properties (see Table 6.2.1). Specifically, the MWCNTs with the lowest outer diameters (CNT-2 and CNT-3) showed smaller Z_{ave}, especially for the calibration dispersions. Moreover, CNT-4 nanotubes, with the largest initial outer diameters, resulted in the lowest polydispersity index values for the calibration and batch dispersions, and they

formed agglomerates with similar sizes to those of the rest of MWCNTs in the batch dispersions. In addition, CNT-4 nanotubes did not show polydispersity index values increase during the ecotoxicity tests. These facts suggest that larger-diameters produce more stable dispersions, which may affect their toxic effects (see *Results and Discussion*-D. magna *acute immobilization tests*), and is consistent with the results obtained by Lin et al.,[13] who demonstrated that MWCNTs with smaller outer diameters had lower potential to be dispersed and stabilized in the presence of humic acids. The different lengths of the MWCNTs studied did not show a clear influence on the stability of the dispersions analyzed.

Concerning the variation of dynamic light scattering parameters throughout the duration of the tests, a clear trend was not observed. However, in the case of CNT-4 nanotubes a decrease in Z_{ave} was found after 48 h and their polydispersity index value remained constant. These results were consistent with those obtained at characterization by UV/Vis spectroscopy (as detailed below).

Characterization by UV/Vis spectroscopy

The fact that the agglomerates diameters influence the UV/Vis absorbances is generally accepted. If the agglomerate sizes are comparable to the light wavelength of the measurements, the intrinsic properties of MWCNTs are the main influencing factor on UV/Vis absorption. However, the agglomerate sizes are the main influencing factor if they are much larger than the wavelength, and poorly dispersed MWCNT agglomerates have a decreased apparent absorption coefficient.[37] The measurement wavelength in the present study was 530 nm, and agglomerates were quite a bit larger for batch dispersions. Therefore, Z_{ave} should be directly related to UV/Vis absorbances. However, the negative correlation expected between both parameters was only observed in the case of the calibration dispersions (Figure 6.2.4). The linear-least square fits were not represented, because lower agglomerate diameters did not result in increased absorbances in all cases, and the statistical spread was considerable.

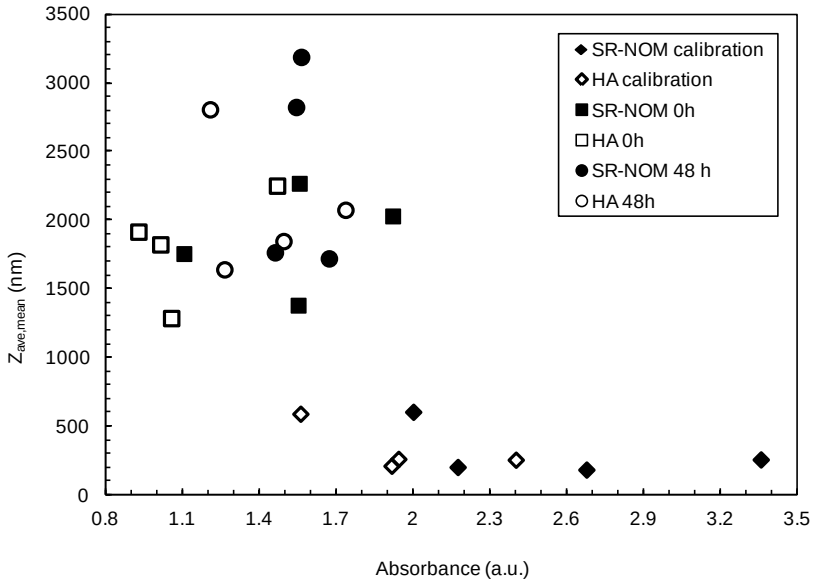

Figure 6.2.4 Correlation between Z_{ave} and absorbance results obtained for calibration and batch dispersions of MWCNTs. $Z_{ave,mean}$ = mean average Z diameter; a.u. = arbitrary unit; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

The simulation of realistic environments involved that batch dispersions were greatly unstable, and the comparison of absorbance and agglomerate size measurements sometimes led to contradictory conclusions. This could be explained by the fact that the UV/Vis absorptions of the batch dispersions might be altered by the sonication of MWCNTs with the salts present in the culture medium. Furthermore, the exudates released by daphnids to mitigate the stress induced during the exposure period[43] might have absorbed in the peak wavelength range of carbon nanotubes, thus interfering with their UV/Vis absorption. Nevertheless, the UV/Vis results were meaningful to assess the dispersion capability of the organic matters used, and overall, SR-NOM produced better and more uniform absorbance results than humic acid, as observed for the dynamic light scattering characterization. The same behavior was found for the dispersions prepared for calibration curves, in which higher values were obtained particularly for UV/Vis absorbances of SR-NOM dispersions (Figures 6.2.4 to 6.2.7).

Calibration standards were made by diluting the 50 mg/L MWCNTs dispersions with 20 mg/L HA and SR-NOM solutions prepared in ultrapure water, obtaining eleven

[43] Handy RD, van den Brink N, Chappell M, et al. **2012**. Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: What have we learnt so far? *Ecotoxicology* 21: 933-972.

levels more: 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5 and 0.1 mg/L. The obtained absorbance values for each concentration are specified in Figures 6.2.5 and 6.2.6.

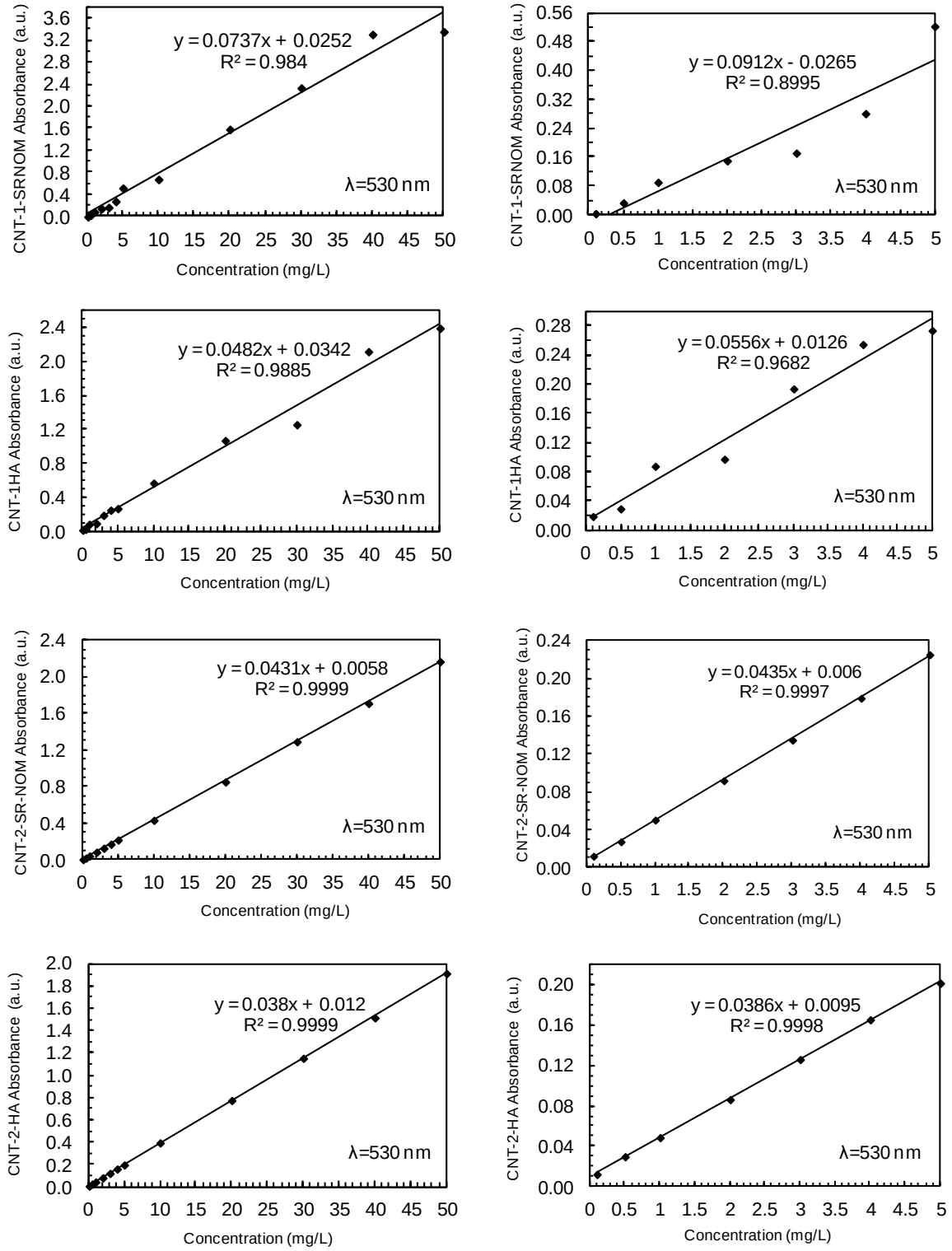

Figure 6.2.5 Calibration curves obtained from absorbance of CNT-1 and CNT-2 dispersions with HA and SR-NOM, in different ranges of dilution levels (0.1 to 50 mg/L). The straight lines are linear least-squares fit to the data.

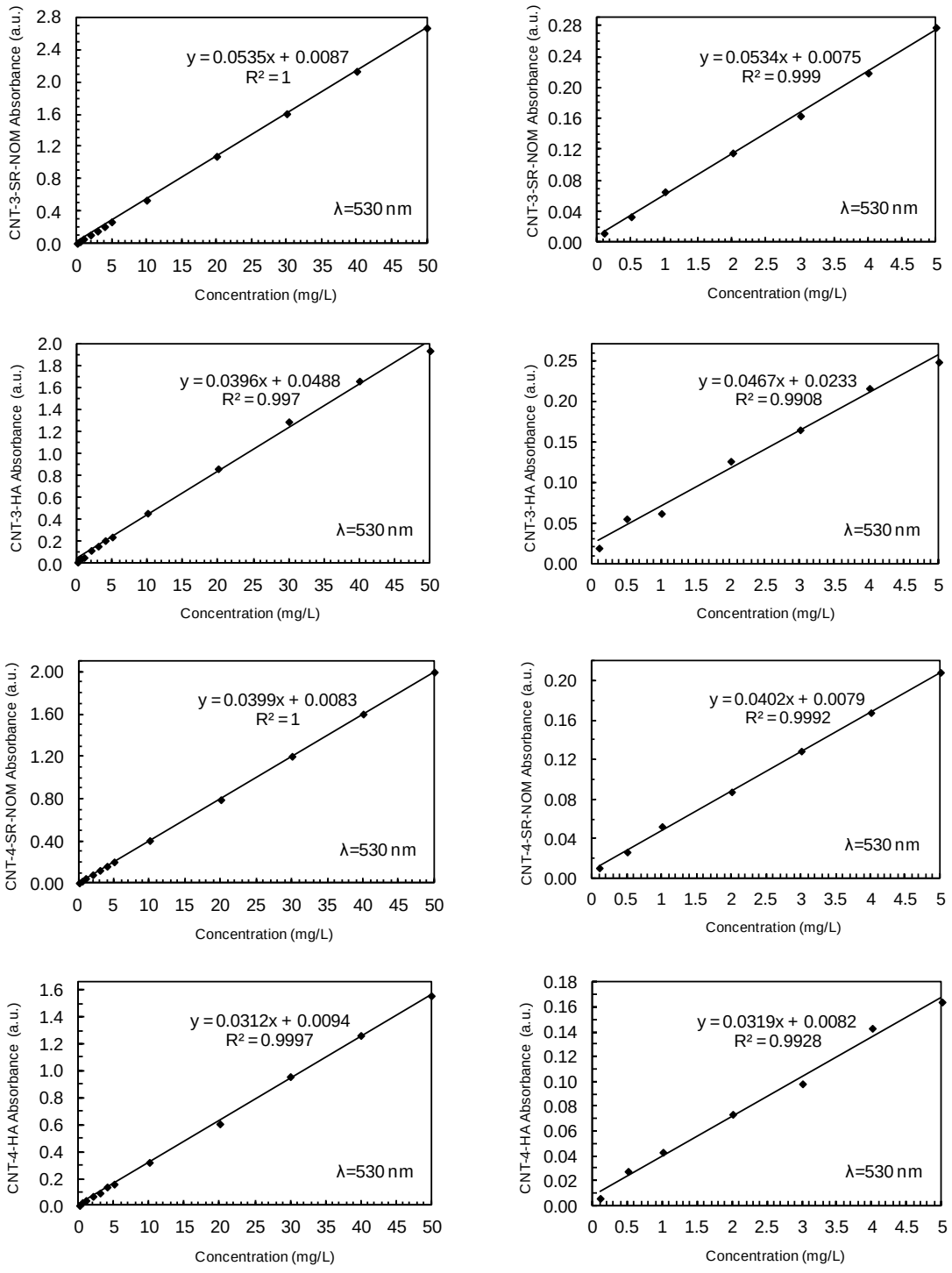

Figure 6.2.6 Calibration curves obtained from absorbance of CNT-3 and CNT-4 dispersions with HA and SR-NOM, in different ranges of dilution levels (0.1 to 50 mg/L). The straight lines are linear least-squares fit to the data.

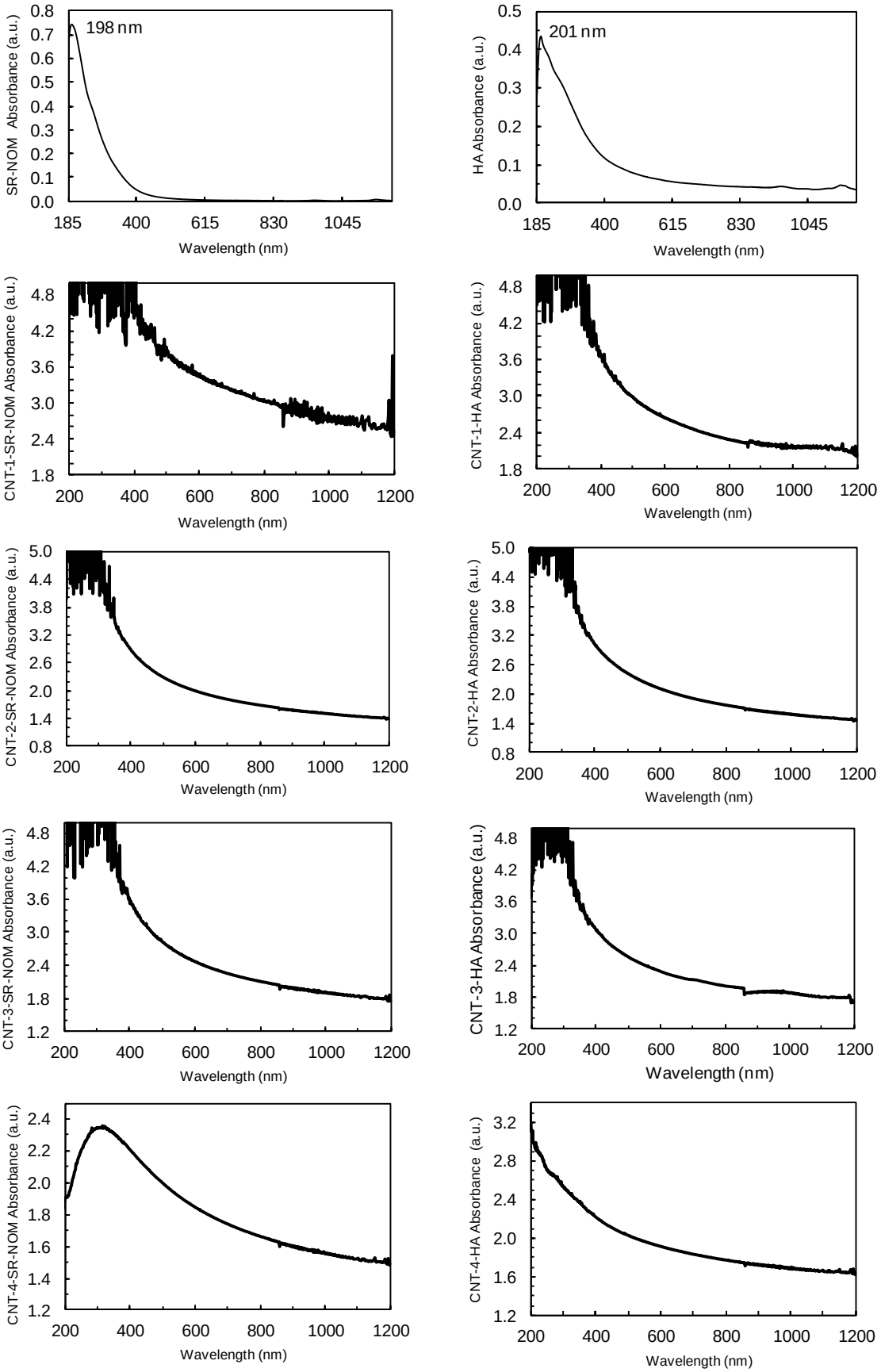

Figure 6.2.7 UV/Vis spectra of MWCNTs dispersions considering HA and SR-NOM as background solution.

The apparent concentrations of MWCNTs in batch dispersions are shown in Table 6.2.4. Generally, they were lower than their corresponding nominal concentrations in calibration dispersions (50 mg/L), given the reduction in their stability promoted by the use of culture medium in their preparation. The apparent concentrations at the beginning of the tests were higher for SR-NOM. However, after 48 h, the opposite effect was observed, because the dispersions prepared with humic acid presented higher concentrations in all cases. Regarding the values obtained for the MWCNTs studied, the highest absorbances corresponded to CNT-4. This result was in accordance with dynamic light scattering characterization, because CNT-4 dispersions showed an increase in stability and the lowest polydispersity index. In the case of humic acid dispersion with CNT-4, a notable increase of absorbance occurred, corresponding to an apparent concentration even higher than the initial 50 mg/L.

Table 6.2.4 Apparent MWCNTs concentrations in batch dispersions at the beginning and end of the ecotoxicity tests, obtained by UV/Vis absorbance measurements.

		CNT-1		CNT-2		CNT-3		CNT-4	
		SR-NOM	HA	SR-NOM	HA	SR-NOM	HA	SR-NOM	HA
Absorbance (a.u.)	0 h	1.5491	1.0135	1.1025	0.9278	1.5537	1.0560	1.9188	1.4699
	48 h	1.5621	1.2078	1.4616	1.4950	1.5443	1.2639	1.6727	1.7372
Apparent concentration (mg/L)	0 h	20.68	20.32	25.45	24.10	28.88	25.43	47.88	46.81
	48 h	20.85	24.35	33.78	39.03	28.70	30.68	41.71	55.38

CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

Considering the behavior of dispersions throughout the duration of the tests, a general increase of MWCNTs absorbances was observed after 48 h for both SR-NOM and humic acid dispersions. As previously observed, this finding was consistent with the decrease in Zave found at the end of the tests for CNT-4. The accumulation and processing of MWCNTs by daphnids might alter the agglomeration state of the dispersions, and Edgington et al.[24] reported disaggregation of MWCNTs in the gut tract of *D. magna*. Conversely, the uptake of the nutrient salts by the organisms could pose a stabilization of MWCNTs during the test, as well as slight pH variations (see *Results and Discussion*-D. magna *acute immobilization tests*).

Characterization by SEM

The imaging conducted (Figures 6.2.8, 6.2.9 and 6.2.10) suggests that SR-NOM and humic acid were adsorbed on the carbon nanotubes. The absorption of organic matter on MWCNTs has been previously demonstrated by means of several techniques.[11,24] We found in some of the images that the adsorption of SR-NOM on nanotubes was higher than that of humic acid, for both calibration and batch dispersions. Natural organic matter adsorbed onto the nanotubes surfaces was observed in Figures 6.2.8A, 6.2.8C, 6.2.8G, 6.2.9A, 6.2.10A, and 6.2.10E (indicated by white arrows), whereas only Figure 10H showed clearly the presence of humic acid. This fact could explain the trend observed by dynamic light scattering and UV/Vis characterization, which indicated lower agglomerate sizes and more stability and uniform results for SR-NOM.

The SEM images of calibration dispersions (Figure 6.2.8) showed a greater homogeneity than the batch dispersions (Figures 6.2.9 and 6.2.10). This enhanced homogeneity was observed, for instance, in the CNT-free areas present in Figure 6.2.8C, 6.2.8D, and 6.2.8E. Considering the high magnification of the imaging, these areas gave an indication of the presence of smaller agglomerates. These observations supported the quite lower polydispersity index values, Z_{ave}, and higher UV/Vis absorbance obtained in the previous characterization for the calibration dispersions.

The differences observed in dynamic light scattering and UV/Vis characterization between the types of nanotubes studied were supported by the SEM imaging. The MWCNTs with the lowest outer diameters for the bulk materials (CNT-2 and CNT-3) showed a decrease in Z_{ave}, which corresponded to MWCNTs better dispersed in SEM images, especially for the calibration dispersions (see Figure 6.2.8C, 6.2.8E). Moreover, the low polydispersity index values of CNT-4 were explained by a uniform dispersion of nanotubes observed in Figures 6.2.8G, 6.2.8H, 6.2.9G, 6.2.9H, 6.2.10G and 6.2.10H. The fact that the agglomerates sizes of CNT-4 were similar to those of the rest of the MWCNTs for the batch dispersions was also observed in SEM imaging, considering their larger outer diameters and the lower magnification required to visualize them. The higher absorptions and apparent concentrations obtained with UV/Vis spectroscopy for CNT-4 were also in accordance with the homogeneous dispersions observed by SEM.

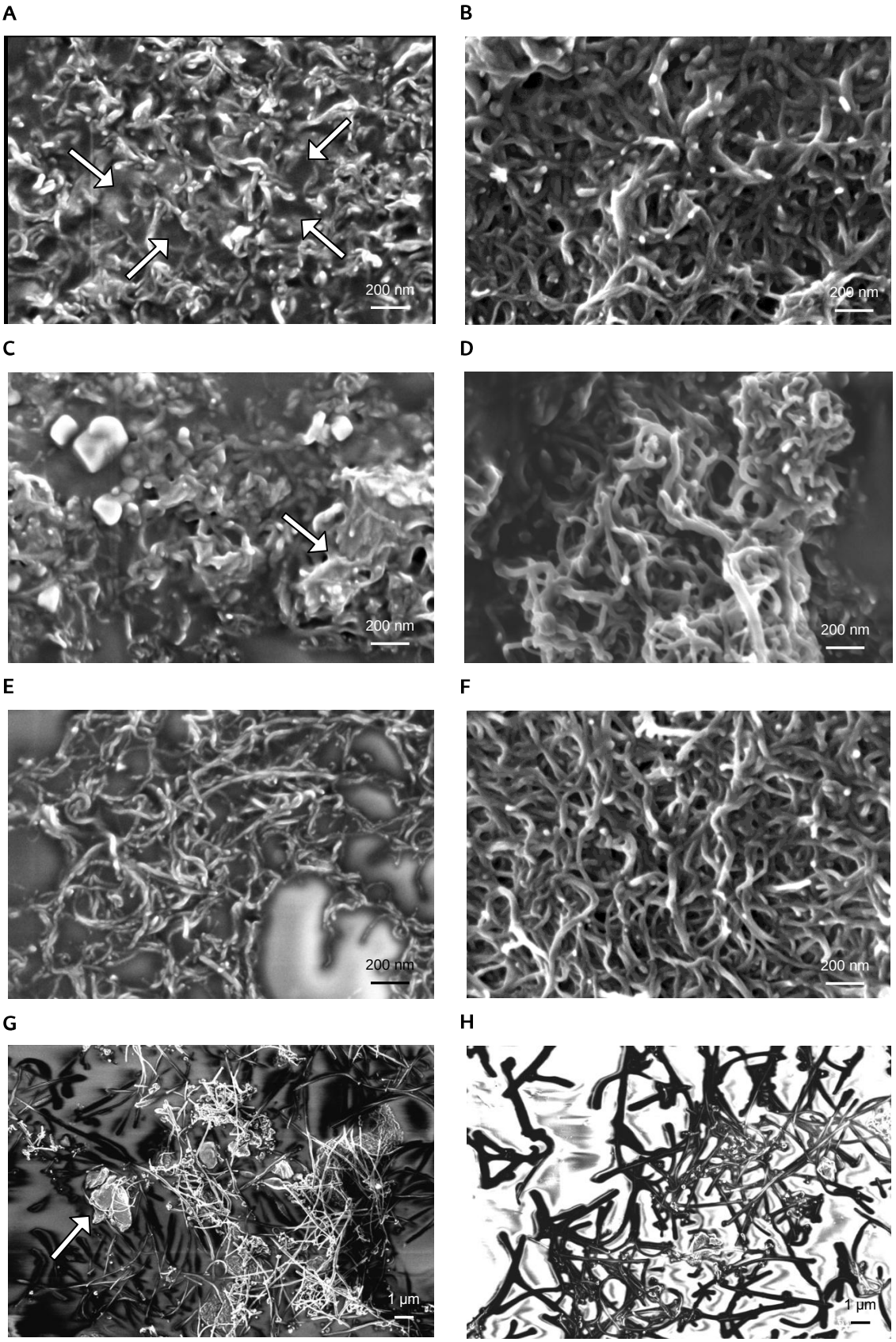

Figure 6.2.8 Scanning electron microscope images of the 50 mg/L multiwalled carbon nanotube calibration dispersions: (A) CNT-1- SR-NOM, (B) CNT-1-HA, (C) CNT-2-SR-NOM, (D) CNT-2-HA, (E) CNT-3-SR-NOM, (F) CNT-3-HA, (G) CNT-4-SR-NOM, (H) CNT-4-HA. CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

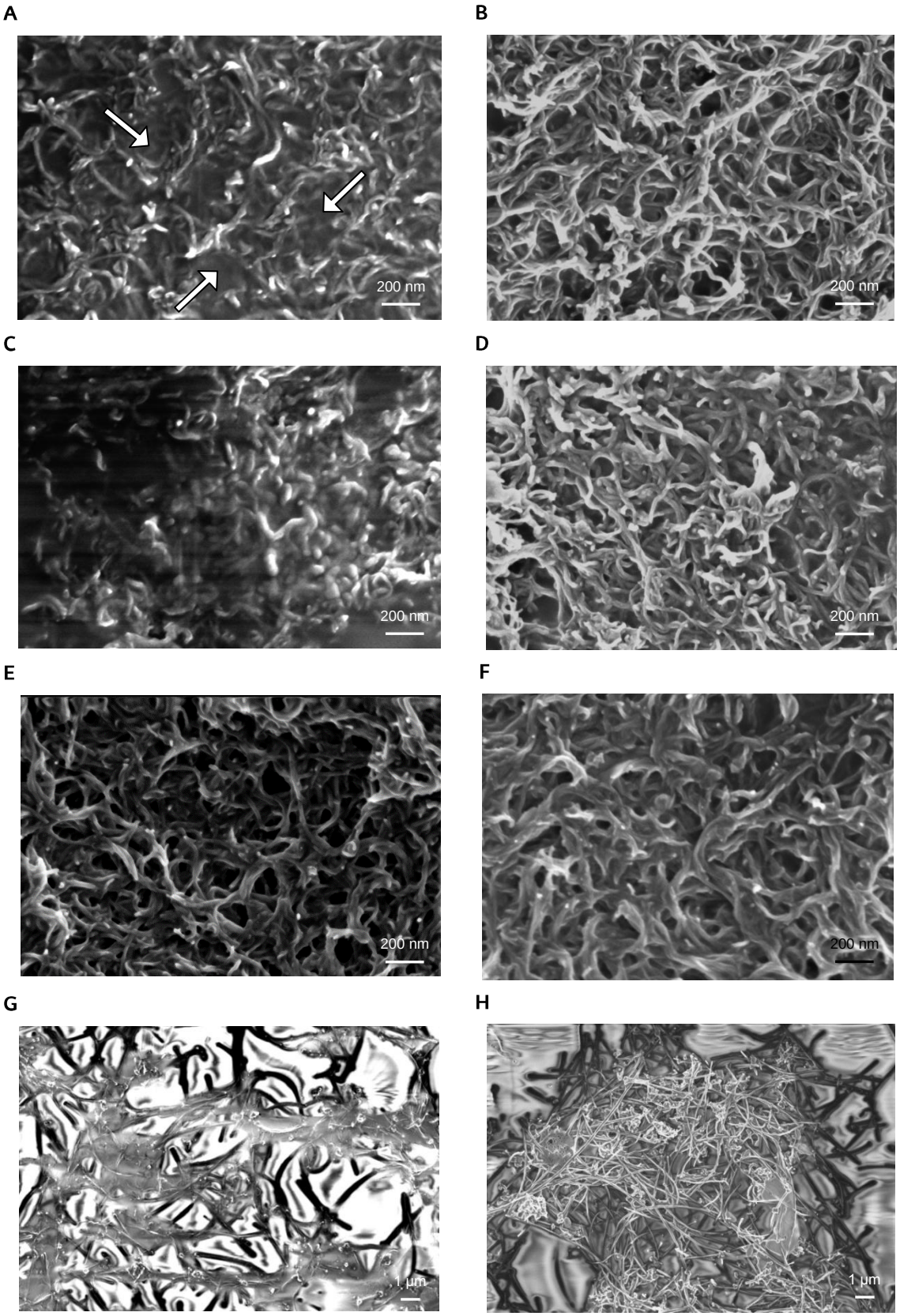

Figure 6.2.9 Scanning electron microscope images of the 50 mg/L multiwalled carbon nanotube batch dispersions at the beginning of the tests: (A) CNT-1-SRNOM 0 h, (B) CNT-1-HA 0 h, (C) CNT-2-SR-NOM 0 h, (D) CNT-2-HA 0 h, (E) CNT-3-SR-NOM 0 h, (F) CNT-3-HA 0 h, (G) CNT-4-SR-NOM 0 h, (H) CNT-4-HA 0 h. CNT = carbon nanotube; SR-NOM =v Suwannee River natural organic matter; HA = humic acid.

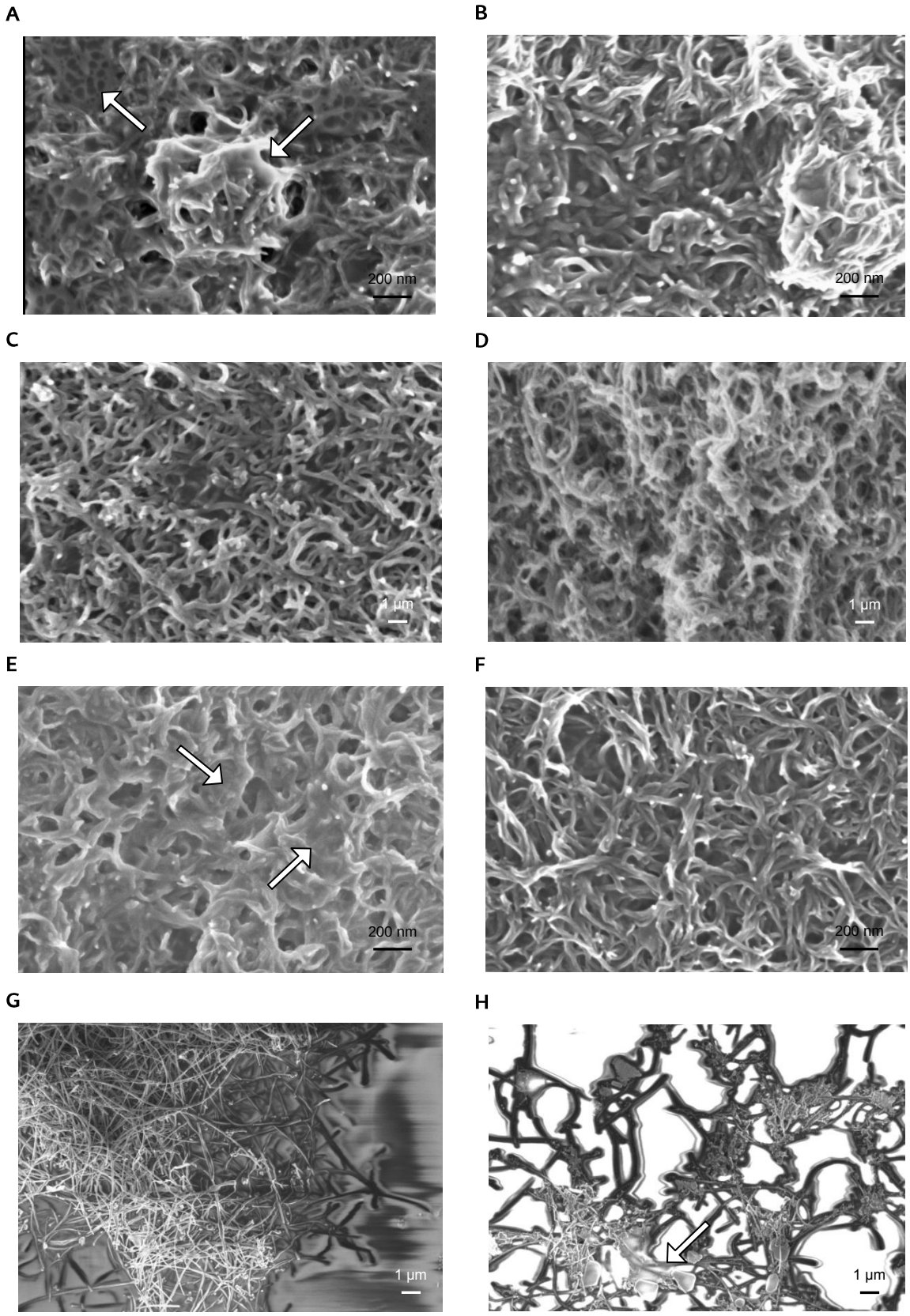

Figure 6.2.10 Scanning electron microscope images of the 50 mg/L multiwalled carbon nanotube batch dispersions at end of the tests: (A) CNT-1-SR-NOM 48 h, (B) CNT-1-HA 48 h, (C) CNT-2-SR-NOM 48 h, (D) CNT-2-HA 48 h, (E) CNT-3-SR-NOM 48 h, (F) CNT-3-HA 48 h, (G) CNT-4-SR-NOM 48 h, (H) CNT-4-HA 48h. CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

The decrease in Z_{ave} for CNT-4 and the overall increase in MWCNT apparent concentrations found after 48 h for both SR-NOM and humic acid batch dispersions were not clearly observed in SEM images (Figures 6.2.8, 6.2.9 and 6.2.10). That SEM images can only show a tiny area of the samples and different agglomerate sizes in the same dispersion can be found is well known. Moreover, the preparation of SEM samples involves changes in the ultimate disposition of nanotubes.

Even though the batch dispersions prepared were greatly unstable, and the comparison between dynamic light scattering and UV/Vis spectroscopy measurements led to contradictory conclusions in some cases, SEM images supported the overall findings and insights obtained by the previous characterization.

6.2.3.2. *D. magna* acute immobilization tests

Given the variation of the apparent concentrations of MWCNTs in the batch dispersions observed during the tests, the initial concentrations of MWCNTs (50 mg/L) were selected to calculate EC20 and EC50. The dissolved oxygen measured in the controls and the batch dispersions was higher than 3 mg/L, in compliance with the validity criteria of the Organisation for Economic Co-operation and Development Guideline 202.[41] The pH values at the end of the tests decreased slightly from the initial 8.3 to average values of 7.9, and they were kept in the range of the performance criteria. Two additional tests were carried out with the reference chemical $K_2Cr_2O_7$, and the EC50 values after 24 h of exposure were 1.11 mg/L and 0.96 mg/L, respectively (in the validation range of 0.6-2.1 mg/L). The 50% immobilization rates were not achieved for most of the dispersions tested. Thus, the EC20 values were used to analyze their effects on daphnids.

The results shown in Table 6.2.5 indicate that MWCNT dispersions prepared with SR-NOM exhibited greater toxicity levels than dispersions prepared with humic acid, taking into account that these two types of organic matter themselves did not cause inhibitory effects. This result was in accordance with the characterization conducted, which showed more stability for SR-NOM dispersions during the tests, and increased stability is assumed to lead to higher toxicological outcomes.[24,31] Specifically, for SR-NOM, the greater stability of the CNT-4 dispersions also contributed to confirm this assumption. The CNT-4s showed lower Z_{ave} with respect to the initial outer diameters, a decrease in the polydispersity index values, higher UV/Vis absorbance, and greater homogeneity in dispersions in the SEM images, compared with the rest

of the MWCNTs. From a physicochemical perspective, the reason for the enhanced stability and increased toxicity with SR-NOM might be related to its nonhumic portion, which contains aliphatic carbon and nitrogen, including carboxylic acids, carbon hydrates, tannic acids, and proteins.[44] Wang et al.[27] reported that the key driving force for the sorption of NOM to MWCNTs were these alkyl (aliphatic) components rather than the aromatic ones of humic acid. Edgington et al.[24] also observed differences in the acute toxicity of MWCNTs to *D. magna*, depending on the sources of the NOM used, but they could not justify their results from either the suspensions or the NOM characterization. The nonhumic portion mentioned previously could be an influencing factor on the variations in acute toxicity that they obtained.

Table 6.2.5 Effective concentration values and lower and upper 95% confidence limits (CL), of MWCNTs dispersions (mg/L) for *Daphnia magna* neonates during 48 h.

Dispersant	Sample	EC20 (95% CL)	EC50 (95% CL)
SR-NOM	CNT-1	4.03 (3.65-4.45)	›50
	CNT-2	2.94 (2.60-3.31)	›50
	CNT-3	ND	ND
	CNT-4	1.08 (0.86-1.35)	27.05 (21.47-34.08)
	SR-NOM	›20	›20
HA	CNT-1	333.15 (315.66-351.60)	››50
	CNT-2	ND	ND
	CNT-3	ND	ND
	CNT-4	ND	ND
	HA	›20	›20

EC20, EC50 = effective concentrations causing 20% and 50% immobilization, respectively; CL = confidence limit; ND = not determined (effective concentration values could not be calculated because of the low toxicity levels and the scattered points obtained in the dose-response curves); SR-NOM = Suwannee River natural organic matter; CNT = carbon nanotube; HA = humic acid.

Furthermore, the current literature has reported the influence of the outer diameter, length, and rigidity of MWCNTs in their potential toxicity. Diameters of 50 nm have shown in vivo and in vitro effects, whereas thicker diameters (150 nm) or tangled (2–

[44] Grillo R, Rosa AH, Fraceto LF. **2015**. Engineered nanoparticles and organic matter: A review of the state-of-the-art. *Chemosphere* 119: 608-619.

20 nm) are less toxic.[4] Conversely, the contribution of the amounts of metal impurities to the toxicity of MWCNTs also has been demonstrated.[4,45] The results obtained in the present study with SR-NOM were fully in accordance with CNT-4 (28-99 nm diameter) showing more adverse effects than the rest of the nanotubes studied (6-24 nm diameter). The CNT-4 nanotubes also had the highest content of impurities (Table 6.2.1). In the case of humic acid dispersions, CNT-4 did not produce toxic effects, which suggested that this type of synthetic organic matter might alter the response of organisms to MWCNTs with respect to that observed in the presence of SR-NOM. Toxicity also might be determined by a combined effect of the outer diameter and length of the carbon nanotubes. Liu et al.[4] and Lanone et al.[5] reported stronger adverse effects induced by the longest CNTs, which was consistent with the EC values and lengths provided in the present study. Considering that the outer diameters of CNT-1, CNT-2, and CNT-3 nanotubes were in similar ranges (Table 6.2.1), their toxic effects decreased with decreasing lengths in the presence of SR-NOM. Carbon nanotube-3, with lengths up to 1300 nm, showed the lowest toxicity; CNT-2, with lengths up to 10000 nm, the highest. Carbon nanotube-1 presented intermediate lengths (up to 5000 nm) and EC values. Nonetheless, CNT-4 length was similar to that of CNT-1 and CNT-2, thus showing that the outer diameter of MWCNTs was a more decisive factor than length in determining the adverse effects on *D. magna*.

Regarding the previously reported endpoints for MWCNTs' ecotoxicity tests with *D. magna*,[3,31,33] lower adverse effects were found in the present study because 48-h EC50 values were not achieved in most cases. This behavior was probably attributable to the fact that, in present study, a more realistic environment was reproduced in the preparation of the dispersions, which led to an increased instability and hence reduced toxicity. Moreover, the preparation of the test dispersions in previous studies generally did not include NOM, which could provide nutritional support to *D. magna*, thus reducing their response to carbon nanotubes. The physical properties of MWCNTs also constitute an important factor affecting their ecotoxicity.[4]

In addition to the characterization of the dispersions at the end of the ecotoxicity tests, optical microscopy was conducted on daphnids to analyze the presence of

[45] Mwangi JN, Wang N, Ingersoll CG, Hardesty DK, Brunson EL, Li H, Deng B. **2012**. Toxicity of carbon nanotubes to freshwater aquatic invertebrates. *Environ Toxicol Chem* 31:1823-1830.

attached MWCNTs agglomerates (Figure 6.2.11). Dead *Daphnia* were selected for the imaging with the aim of visualizing the differences with live organisms in controls and organic matter solutions.

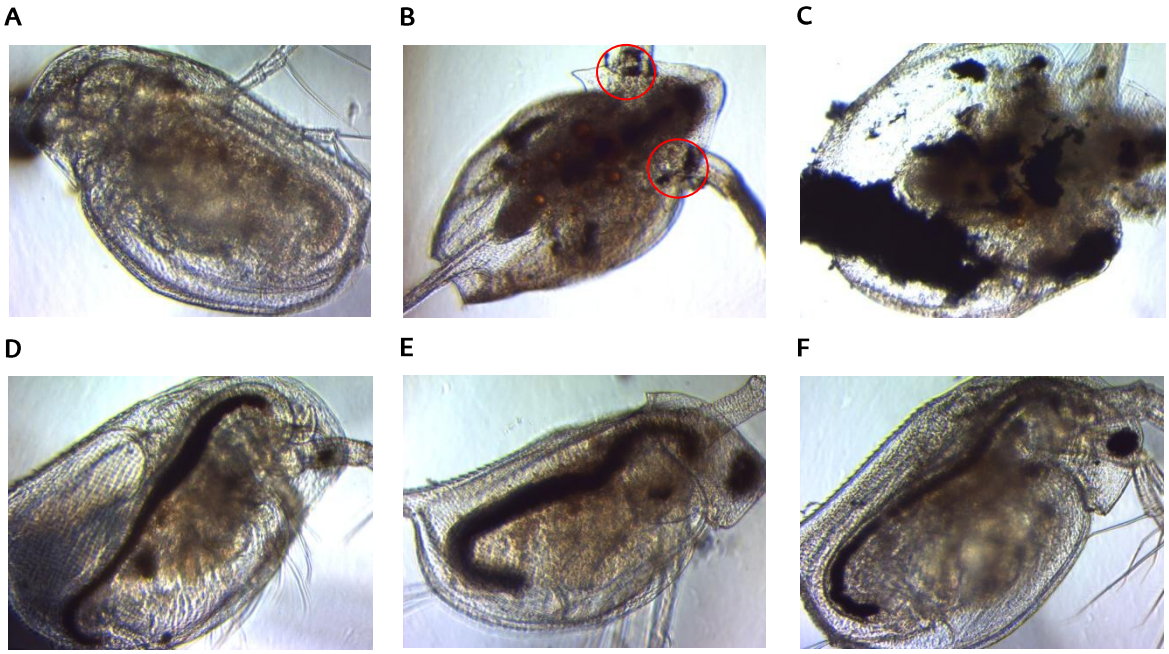

Figure 6.2.11 Optical microscope images of *Daphnia magna* exposed to the multiwalled carbon nanotube dispersions at the end of the tests: (A) Control, (B) Dead *Daphnia* exposed to 50 mg/L CNT-1 in SR-NOM, (C) Dead *Daphnia* exposed to 50 mg/L CNT-4 in SR-NOM, (D) Live *Daphnia* exposed to 20 mg/L HA (background substance), (E) Dead *Daphnia* exposed to 0.5 mg/L CNT-3 in HA, (F) Dead *Daphnia* exposed to 5 mg/L CNT-4 in HA. CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

The dispersions prepared with SR-NOM showed agglomerates of MWCNTs on the body surface (Figure 6.2.11C) and antennae of the organisms (red circles in Figure 6.2.11B), and the accumulation of nanotubes on their external surface has been observed to be a potential mechanism of toxicity.[46] However, in the case of humic acid dispersions, a greater amount of MWCNTs was observed in the digestive tract of daphnids, given their dark coloration (Figure 6.2.11E and 6.2.11F). Nonetheless, the organisms exposed to humic acid alone as background substance (Figure 6.2.11D) also showed this dark coloration. This fact could pose a greater uptake of humic acid by *D. magna* as a food source and thus explain the reduction in immobilization with respect to MWCNTs dispersed in SR-NOM solutions. The key driving force for the sorption of NOM to MWCNTs is the alkyl (aliphatic) components rather than the aromatic ones of humic acid. The driving forces for the adsorption of SR-NOM onto

[46] Roberts AP, Mount AS, Seda B, Souther J, Qiao R, Lin S, Ke PC, Rao AM, Klaine SJ. **2007**. In vivo biomodification of lipid-coated carbon nanotubes by Daphnia magna. *Environ Sci Technol* 41:3025-3029.

MWCNTs might be greater than those for humic acid, thus resulting in different modes of action on daphnids. Humic acid, more loosely adsorbed onto nanotubes, might be used as a nutritional support by daphnids during the test, and this fact may delay the digestion of MWCNTs. Therefore, although MWCNTs would be bioavailable for daphnids, toxicity was not observed during the exposure period.

Although SR-NOM has provided a better capability for the stabilization of MWCNTs, and toxicity results on *D. magna* are consistent with those reported in the literature, further research needs to be conducted in this field. Several key aspects, such as the feeding during assays, have been demonstrated to play an essential role in the toxicity mechanisms of carbon nanotubes toward daphnids.[8] In addition, their adverse effects are exerted to several generations of *D. magna* on their survival, reproduction, and growth.[32] These factors might be considered in future studies to ensure the suitability of SR-NOM for the analysis of the ecotoxicity of MWCNTs toward *D. magna* and other organisms at the base of the food chain.

6.2.4. Conclusions

The characterization performed in the present study indicates that NOM provides an increased stability to the MWCNTs' dispersions with respect to synthetic organic matter. Suwannee River-NOM produced a decrease in Z_{ave} or polydispersity index values for all of the nanotubes studied, and also greater and more uniform UV/Vis absorbance results than humic acid. In addition, SEM imaging indicated a higher adsorption of SR-NOM on nanotubes. The outcomes of the toxicity assays confirmed the previously reported finding that increased stability leads to higher inhibitory effects on *D. magna*, because MWCNTs dispersed with SR-NOM exhibit greater toxicity levels than those dispersed with humic acid. The latter seemed to alter the response of the organisms to carbon nanotubes compared with that shown in the presence of SR-NOM. Furthermore, the results obtained with NOM allowed observing the important role of the outer diameter and content of impurities of MWCNTs in their stability and ecotoxicity on daphnids. Suwannee River-NOM is considered to be more appropriate than Sigma-Aldrich humic acid for the ecotoxicity assessment of MWCNTs, not only because of the stability provided to the dispersions, but also because of its capability of simulating the real conditions in aquatic ecosystems.

6.3. Contribution 3

Towards the standardization of nanoecotoxicity testing: natural organic matter 'camouflages' the adverse effects of TiO_2 and CeO_2 nanoparticles on green microalgae

Science of the Total Environment 2016, 543: 95-104

Contents

Abstract:

In the last few years, the emission of CeO_2 and TiO_2 nanoparticles (NPs) into the environment has been raising concerns about their potential adverse effects on wildlife and human health. Aquatic organisms constitute one of the most important pathways for the entrance of these NPs and transfer throughout the food web, but divergences exist in the experimental data published on their aquatic toxicity. The pressing need for standardization of methods to analyze their ecotoxicity requires aquatic media representing realistic environmental conditions. The present study aimed to determine the usefulness of Suwannee River natural organic matter (SR-NOM) in the assessment of the agglomeration kinetics and ecotoxicity of CeO_2 and TiO_2 NPs towards green microalgae Pseudokirchneriella subcapitata*. SR-NOM alleviated the adverse effects of NPs on algal growth, completely in the case of TiO_2 NPs and partially in the case of CeO_2 NPs, suggesting a 'camouflage' of toxicity. This behavior has been observed also for other algal species and types of natural organic matter in the literature. Furthermore, SR-NOM markedly increased the stability of the NPs in algal medium, which led to a better reproducibility of the toxicity test results, and provided an electrophoretic mobility similar to that previously reported in various river and groundwaters. Thus, SR-NOM can be a representative sample of what is found in many different ecosystems, and the observed 'camouflage' of the effects of CeO_2 and TiO_2 NPs on algal cells might be considered as a natural interaction occurring in their standardized ecotoxicological assessment.*

Keywords: CeO_2; TiO_2; nanoparticles; ecotoxicity; Suwannee river natural organic matter; microalgae.

6.3.1. Introduction

Nanoscale CeO_2 and TiO_2 are two of the most extensively manufactured nanomaterials (MNMs) used currently. They are incorporated into a wide variety of products, including catalysts, gas sensors, solar cells, oxygen pumps, fuels in the automotive industry, paints, coatings and cosmetics.[1,2] Consequently, the constant increase in their large scale production and their inherent emission into the environment are raising concerns regarding their potential adverse effects on wildlife and human health.[3,4]

Aquatic organisms constitute one of the most important pathways for the entrance and transfer of MNMs throughout the food webs in ecosystems.[5] The data published on the aquatic toxicity of CeO_2 and TiO_2 nanoparticles (NPs) are divergent[6,7] and this limited understanding of their impacts poses a barrier to their current and potential applications. Factors such as physico-chemical properties (size, shape and surface chemistry) and environmental conditions (pH, ionic strength, colloids and natural organic matter concentration) play an important role on the fate and toxic effects of these NPs.[3,5,6] The combination of these properties and conditions may result in either their agglomeration or stabilization, affecting their bioavailability and determining their toxicity. If the degree of agglomeration of the NPs in the test media is not representative of that occurring in natural waters, the current regulatory testing can under- or overestimate their toxicity to aquatic organisms,

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[1] Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TS, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR. **2008**. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environ Toxicol Chem* 27:1825-1851.

[2] Yadav T, Mungray AA, Mungray AK. Fabricated Nanoparticles: Current Status and Potential Phytotoxic Threats. Whitacre DM (ed.), *Rev Environ Contam T*, Vol 230. Springer International Publishing, Switzerland, pp 83-110. 2014.

[3] Keller AA, Wang H, Zhou D, Lenihan HS, Cherr G, Cardinale BJ, Miller R, Ji Z. **2010**. Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices. *Environ Sci Technol* 44:1962-1967.

[4] Keller AA, McFerran S, Lazareva A, Suh S. **2013**. Global life cycle releases of engineered nanomaterials. *J Nanopart Res* 15:1692.

[5] Baun A, Hartmann NB, Grieger K, Kusk KO. **2008**. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotoxicology* 17:387-395.

[6] Menard A, Drobne D, Jemec A. **2011**. Ecotoxicity of nanosized TiO_2. Review of in vivo data. *Environ Pollut* 159:677-684.

[7] Booth A, Størseth T, Altin D, Fornara A, Ahniyaz A, Jungnickel H, Laux P, Luch A, Sørensen L. **2015**. Freshwater dispersion stability of PAA-stabilised cerium oxide nanoparticles and toxicity towards Pseudokirchneriella subcapitata. *Sci Total Environ* 505:596-605.

which is considered as a prominent concern within the scientific community.[8] Therefore, the pressing need for standardization of methods to analyze the ecotoxicity of CeO_2 and TiO_2 NPs[9,10] requires media which better represent the behaviour of MNMs in realistic environmental conditions.

The interaction of natural organic matter (NOM) and its predominant substance, humic acid (HA),[11] with MNMs, is an issue extensively analyzed in the literature. NOM may influence the stability and toxicity of MNMs and can also play an important role in removing toxic substances from effluents, but further research is needed to better understand these dynamic interactions.[12] In the case of CeO_2 and TiO_2 NPs, it has been widely demonstrated that different types of NOM promotes their stabilization in aqueous media at typical environmental concentrations[3,13-15] and affect also their toxic effects in aquatic and soil organisms.[10,16,17] Likewise, synthetic organic matters have been proved to interact with these NPs, but the influence on their stability and

[8] Park S, Woodhall J, Ma G, Veinot JGC, Cresser MS, Boxall ABA. **2014**. Regulatory ecotoxicity testing of engineered nanoparticles: are the results relevant to the natural environment? *Nanotoxicology* 8: 583-592.

[9] Savolainen K, Backman U, Brouwer D, Fadeel B, Fernandes T, Kuhlbusch T, Landsiedel R, Lynch I, Pylkkänen L. Nanosafety in Europe 2015-2025: Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations. EDITA: Helsinki, Finland. 2013.

[10] Van Hoecke K, De Schamphelaere KAC, Van der Meeren P, Smagghe G, Janssen CR. **2011**. Aggregation and ecotoxicity of CeO_2 nanoparticles in synthetic and natural waters with variable pH, organic matter concentration and ionic strength. *Environ Pollut* 159:970-976.

[11] Tang WW, Zeng GM, Gong JL, Liang J, Xu P, Zhang C, Huang B. **2014**. Impact of humic/fulvic acid on the removal of heavy metals from aqueous solutions using nanomaterials: a review. *Sci Total Environ* 468-469:1014-1027.

[12] Grillo R, Rosa AH, Fraceto LF. **2015**. Engineered nanoparticles and organic matter: A review of the state-of-the-art. *Chemosphere* 119:608-619.

[13] Yang K, Lin D, Xing B. **2009**. Interactions of Humic Acid with Nanosized Inorganic Oxides. *Langmuir* 25:3571-3576.

[14] Quik JTK, Lynch I, Van Hoecke K, Miermans CJH, De Schamphelaere KAC, Janssen CR, Dawson KA, Cohen Stuart MA, Van de Meent D. **2010**. Effect of natural organic matter on cerium dioxide nanoparticles settling in model freshwater. *Chemosphere* 81:711-715.

[15] Erhayem M, Sohn M. **2014**. Stability studies for titanium dioxide nanoparticles upon adsorption of Suwannee River humic and fulvic acids and natural organic matter. *Sci Total Environ* 468-469:249-257.

[16] Schwabe F, Schulin R, Limbach LK, Stark W, Bürge D, Nowack B. **2013**. Influence of two types of organic matter on interaction of CeO_2 nanoparticles with plants in hydroponic culture. *Chemosphere* 91:512-520.

[17] Collin B, Oostveen E, Tsyusko OV, Unrine JM. **2014**. Influence of Natural Organic Matter and Surface Charge on the Toxicity and Bioaccumulation of Functionalized Ceria Nanoparticles in Caenorhabditis elegans. *Environ Sci Technol* 48:1280-1289.

ecotoxicity is not as clear as that of NOM.[16,18,19] Currently, several types of NOM are commercially available and their key advantage over laboratory-synthesized substances is a more realistic simulation of the ecosystems in the toxicity assays.

NOM and HA from Suwannee River[20] are the most analyzed organic matters in the study of bioavailability of CeO_2 and TiO_2 NPs.[7,21-30] A recent research on nano-TiO_2 stability upon adsorption of Suwannee River humic substances concluded that Suwannee River NOM (SR-NOM) was the most representative sample of what is found naturally and would likely provide the most useful outcomes.[15] Hence, SR-NOM might fulfill the need for standardization mentioned above. Nonetheless, further research is still required to prove its suitability in the ecotoxicological assessment of MNMs. As reported by us elsewhere,[31] SR-NOM provides an increased

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[18] Zhu M, Wang H, Keller AA, Wang T, Li F. **2014**. The effect of humic acid on the aggregation of titanium dioxide nanoparticles under different pH and ionic strengths. *Sci Total Environ* 487:375-380.

[19] Wang H, Burgess RM, Cantwell MG, Portis LM, Perron MM, Wu F, Ho KT. **2014**. Stability and aggregation of silver and titanium dioxide nanoparticles in seawater: role of salinity and dissolved organic carbon. *Environ Toxicol Chem* 33:1023-1029.

[20] IHSS (International Humic Substances Society)
Website: http://www.humicsubstances.org/ Last access: april 2015

[21] Li K, Chen Y. **2012**. Effect of natural organic matter on the aggregation kinetics of CeO_2 nanoparticles in KCl and $CaCl_2$ solutions: measurements and modeling. *J Hazard Mater* 209-210:264-270.

[22] Quik JTK, Stuart MC, Wouterse M, Peijnenburg W, Hendriks AJ, Van de Meent D. **2012**. Natural colloids are the dominant factor in the sedimentation of nanoparticles. *Environ Toxicol Chem* 31:1019-1022.

[23] Thio BJR, Zhou D, Keller AA. **2011**. Influence of natural organic matter on the aggregation and deposition of titanium dioxide nanoparticles. *J Hazard Mater* 189:556-563.

[24] Chowdhury I, Cwiertny DM, Walker SL. **2012**. Combined Factors Influencing the Aggregation and Deposition of nano-TiO_2 in the Presence of Humic Acid and Bacteria. *Environ Sci Technol* 46:6968-6976.

[25] Loosli F, Le Coustumer P, Stoll S. **2013**. TiO_2 nanoparticles aggregation and disaggregation in presence of alginate and Suwannee River humic acids. pH and concentration effects on nanoparticle stability. *Water Res* 47:6052-6063.

[26] Yang SP, Bar-Ilan O, Peterson RE, Heideman W, Hamers RJ, Pedersen JA. **2013**. Influence of Humic Acid on Titanium Dioxide Nanoparticle Toxicity to Developing Zebrafish. *Environ Sci Technol* 47:4718-4725.

[27] Cupi D, Hartmann NB, Baun A. **2015**. The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide, and titanium dioxide nanoparticles with Daphnia magna. *Environ Toxicol Chem* 34:497-506.

[28] Dasari TP, Hwang HM. **2013**. Effect of humic acids and sunlight on the cytotoxicity of engineered zinc oxide and titanium dioxide nanoparticles to a river bacterial assemblage. *J Environ Sci* 25:1925–1935.

[29] Neale PA, Jämting AK, O'Malley E, Herrmann J, Escher BI. **2015**. Behaviour of titanium dioxide and zinc oxide nanoparticles in the presence of wastewater-derived organic matter and implications for algal toxicity. *Environ Sci: Nano* 2:86-93.

[30] Mwaanga P, Carraway ER, Schlautman MA. **2014**. Preferential sorption of some natural organic matter fractions to titanium dioxide nanoparticles: influence of pH and ionic strength. *Environ Monit Assess* 186:8833-8844.

[31] Cerrillo C, Barandika G, Igartua A, Areitioaurtena O, Uranga N, Mendoza G. **2015**. Colloidal stability and ecotoxicity of multiwalled carbon nanotubes: Influence of select organic matters. *Environ Toxicol Chem* In press. DOI: 10.1002/etc.3172.

colloidal stability to multiwalled carbon nanotubes with respect to synthetic HA, which resulted in higher adverse effects on *Daphnia magna*. However, Cupi et al.[27] observed that the addition of SR-NOM alleviated Ag NPs toxicity towards *Daphnia magna*, and caused agglomeration and settling of TiO_2 NPs in their culture medium. They highlighted the lack of studies that systematically investigate the stability of NP dispersions in the presence of NOM and its implications in the toxicity tests outcome, and suggested that SR-NOM should be added only in certain cases. This approach for the standardization of toxicity testing on a case-by-case basis for every possible exposure scenario has been supported also in other studies.[28] Although alternative testing strategies have proposed a more efficient assessment of the risks of MNMs,[32] the current lack of specific tools to identify and predict them makes necessary a comprehensive ecotoxicological assessment of the growing number of MNMs so far, at least for various trophic levels.

Considering that the selection of reference materials and methods to assess the ecotoxicity of CeO_2 and TiO_2 NPs still remains unsolved, the present study aims to serve as a next step towards the establishment of standardized ecotoxicity tests of these nanomaterials. The agglomeration kinetics and ecotoxicity of CeO_2 and TiO_2 NPs towards *Pseudokirchneriella subcapitata* were analyzed in the presence and absence of SR-NOM. These unicellular green algae were selected considering their key role in the aquatic ecosystems and in regulatory testing. The standardization approach of this work also included the calculation of the dispersion parameters required to optimize the energy delivered to the NPs during the preparation of the dispersions.

6.3.2. Materials and Methods

6.3.2.1. Chemicals and materials

CeO_2 and TiO_2 nanoparticles were acquired in powdered form from JRC (Joint Research Centre-European Commission) Repository. Their primary characterization data (Table 6.3.1) were also provided by JRC.

[32] Stone V, Pozzi-Mucelli S, Tran L, Aschberger K, Sabella S, Vogel U, et al. **2014**. ITS-NANO - Prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. *Part Fibre Toxicol* 11:9.

Table 6.3.1 Physical descriptions of the NPs studied.

Nanomaterial	Supplier identification	Size (nm)[a]	Surface area (m^2/g) [b]	Impurities (wt %)
CeO_2	JRCNM02102a	33-49	28	‹0.1%
TiO_2	JRCNM01003a	22-27	51	4.1%

[a] Transmission electron microscope (TEM) data on the primary particle size

[b] Obtained by Branauer-Emmett-Teller (BET) analysis

Standard Suwannee River NOM (SR-NOM) obtained from the International Humic Substances Society (IHSS)[20] was used as a model NOM without any further purification.

All stock dispersions and solutions were prepared in ultrapure water, produced by a Milli-Q water filtration system (Millipore). The rest of chemicals used were p.a. grade and obtained from Sigma-Aldrich and Scharlab.

6.3.2.2. Preparation of NP dispersions

The stock dispersions were obtained by adding 10 mL of Milli-Q water to 25.6 mg CeO_2/TiO_2 NPs in 20 mL glass scintillation vials and then sonicating with an ultrasonic homogenizer, a widely accepted method that ensures reasonable stability.[3,27] It has shown to provide better optimization of the energy delivered to the MNMs than other devices, such as ultrasonic baths.[33] The sonicator (VIBRACELL-VCX750, SONICS&MATERIALS) operated with a standard probe (136 mm length and 13 mm diameter), at a frequency of 20 kHz, continuous mode for 12 minutes and output power fixed at 750 W at 20% amplitude. The calorimetric method described by Taurozzi et al.[34] was used to calculate the sonication time and amplitude required to obtain agglomerate sizes as near as possible to the nanometric range.

A 600 mL borosilicate glass beaker was filled with 500 mL thermally equilibrated Milli-Q water, in order to calculate the energy delivered by the sonication method. Its temperature and mass were measured with an uncertainty of ±0.1 °C and ±0.1 g, respectively. The beaker was placed in the sonicator chamber and the tip was immersed to a position 2.5 cm below the liquid surface. The temperature probe was

[33] Cerrillo C, Barandika G, Igartua A, Areitioaurtena O, Marcaide A, Mendoza G. **2015**. Ecotoxicity of multiwalled carbon nanotubes: Standardization of the dispersion methods and concentration measurements. *Environ Toxicol Chem* 34:1854-1862.

[34] Taurozzi JS, Hackley VA, Wiesner, MR. **2011**. Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment-issues and recommendations. *Nanotoxicology* 5:711-729.

mounted (using a clamp) at 2.5 cm depth and 1 cm away from the sonicator probe. The sonicator output selected was 20% amplitude (considering previous dispersion tests carried out in our laboratory), operating in continuous mode. The temperature increase of the water was recorded for 6.5 minutes with a time resolution of 30 seconds.

The calculation of the delivered acoustic energy was performed obtaining the best linear fit (R^2›0.990) between the measured temperature and time using least squares regression. The effective delivered power was determined using the following equation:

$$P = \frac{\mathrm{d}T}{\mathrm{d}t} M C_p$$

where *P* is the delivered acoustic power (W), *dT/dt* is the slope of the regression curve, *M* is the mass of liquid (g), and C_p is the specific heat of the liquid ($J \cdot g^{-1} \cdot {}^{o}C^{-1}$).

The effective delivered acoustic power (*P*) was 11.76 W. The linear fits between the measured temperature as function of time using least squares regression are represented in Figure 6.3.1.

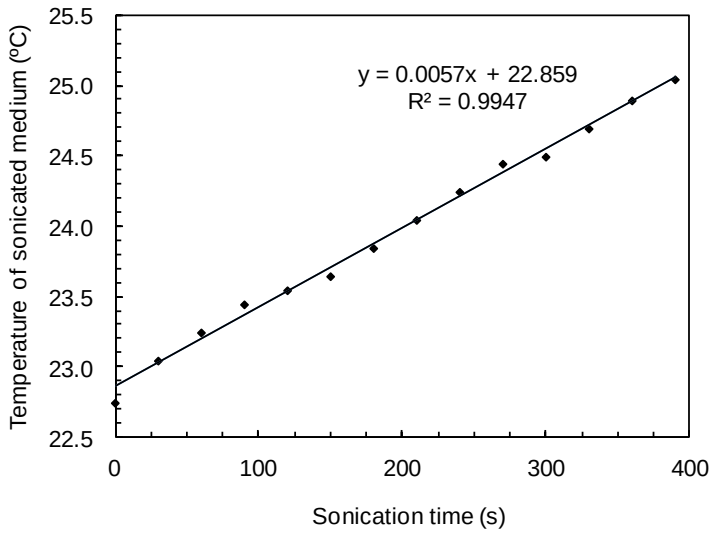

Figure 6.3.1 Linear fits between the measured temperature as function of time sonicator probe.

The total amount of energy delivered was obtained considering the applied power and also the total amount of time that the dispersion was subjected to the ultrasonic treatment:

$$E = P \times t$$

where *E* is the total amount of energy (J), *P* is the delivered acoustic power (W) and *t* is the total amount of time (s).

A sonication time of 12 min was selected taking into account previous dispersion tests carried out in our laboratory, and the total amount of energy delivered (*E*) was 8.467 J. Thus, the acoustic energy delivered by the probe was optimized and enabled to obtain agglomerate sizes as near as possible to the nanometric range. It was important to consider that the actual volumes and temperatures of NPs dispersions were different from that used in the calculation of the energy delivered by the sonication methods. However, this aspect was noted in the calorimetric method,[34] since it was simply intended to allow the reporting and transference of sonication power levels between users, but not to measure the actual fraction of power utilized for powder disruption under specific dispersion conditions.

During sonication, the vials were held in an ice bath to minimize rising of the temperature of the sample, and the probe was inserted between the upper quarter and upper half of the dispersion volume. These conditions maximized the liquid-probe surface area exposed to the acoustic waves, and the vial wall surface/volume ratio for dissipation of heat by the cooling bath.[34] Both CeO_2 and TiO_2 NP dispersions were prepared in the same manner and delivered with the same concentration in order to ensure the consistency of test procedures. The high initial concentrations (2560 mg/L) were selected to perform subsequent dilution into the algae growth medium for conducting the ecotoxicity tests, according to the Technical Guidance Document developed in the EU FP7 NANoREG Project.[35] The characterization and ecotoxicological assessment of the NP dispersions was conducted immediately after their preparation.

6.3.2.3. Algae ecotoxicity studies

The ecotoxicity of CeO_2 and TiO_2 NPs towards unicellular green algae *Pseudokirchneriella subcapitata* was determined in the presence and absence of SR-NOM, according to the OECD Guideline 201 "Algal growth inhibition test".[36] The algal cells were obtained from the CCAP (Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, UK). The tests were conducted in the form of range-finding pre-tests to observe the effects of SR-NOM on a wide range of NP

[35] KA Jensen. Testing the test in NANoREG: Nanomaterial Characterization and Technical Guidance for Toxicological Testing. 2014. http://echa.europa.eu/news-and-events/events/event-details/-/journal_content/56_INSTANCE_DR2i/title/topical-scientific-workshop-regulatory-challenges-in-risk-assessment-of-nanomaterials Last access: July 2015.

[36] Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD Guideline 201. Organization for Economic Cooperation and Development (OECD). Paris, France. Adopted 23 March 2006, corrected 28 July 2011.

concentrations, since obtaining accurate effective concentration data was not within the aim of the present study. The system response was evaluated as a function of growth of algal cultures exposed to NPs in comparison with the average growth of unexposed control cultures.

Pseudokirchneriella subcapitata culturing was conducted by maintaining them on sloped agar tubes and transferred to fresh agar at least once every two months. In order to adapt the algae to the test conditions and ensure that they were in the exponential growth phase when used in the tests, an inoculum culture was prepared in the OECD growth medium 3 days before the start of the test. The initial biomass concentration in the inoculums culture was adjusted to $5x10^5$ cells/mL to obtain a concentration of $5x10^3$ cells/mL in the volume of the test dispersions (100 mL).

The algal growth medium was prepared by adding an appropriate volume of the stock solutions 1-4 to sterile ultrapure water. The stock solutions of nutrients were prepared according to the Table 6.3.2.

Table 6.3.2 Concentration of nutrients in *Pseudokirchneriella subcapitata* medium.

Stock solution	Nutrient	Concentration in stock solution	Final concentration in test solution
1: macro nutrients	NH_4Cl	1.5 g/L	15 mg/L
	$MgCl_2 \cdot 6H_2O$	1.2 g/L	12 mg/L
	$CaCl_2 \cdot 2H_2O$	1.8 g/L	18 mg/L
	$MgSO_4 \cdot 7H_2O$	1.5 g/L	15 mg/L
	KH_2PO_4	0.16 g/L	1.6 mg/L
2: Fe-EDTA[a]	$FeCl_3 \cdot 6H_2O$	64 mg/L	64 µg/L
3: trace elements	H_3BO_3	185 mg/L	185 µg/L
	$MnCl_2 \cdot 4H_2O$	415 mg/L	415 µg/L
	$ZnCl_2$	3 mg/L	3 µg/L
	$CoCl_2 \cdot 6H_2O$	1.5 mg/L	1.5 µg/L
	$CuCl_2 \cdot 2H_2O$	0.01 mg/L	0.01 µg/L
	$Na_2MoO_4 \cdot 2H_2O$	7 mg/L	7 µg/L
4: bicarbonate	$NaHCO_3$	50 g/L	50 mg/L

[a] $Na_2EDTA \cdot 2H_2O$ was removed to avoid binding on metal ions.

The stock solutions 2 and 4 were sterilized by membrane filtration (mean pore diameter 0.2 µm), and stock solutions 1 and 3 were sterilized by autoclaving (120 °C, 15 min). The solutions were stored in the dark at 4 °C. Algal growth medium was prepared by adding 10 mL of stock solution 1 and 1 mL of stock solution 2, 3 and 4 into a 1 L volumetric flask, and then filling up to 1000 mL with sterilized ultrapure water. The pH was adjusted to 8.3, with either 1 M HCl or 1 M NaOH.

The test dispersions were prepared by transferring the required volume of stock dispersions of NPs into 250 mL Erlenmeyer flasks and adding algae growth medium up to the 100 mL mark. The flasks were capped with air-permeable cellulose stoppers to prevent cross-contamination. Exposures were conducted at nominal NP concentrations of 160, 40, 10, 2.5 and 0.6 mg/L. The first level of the dilution series (160 mg/L) was selected according to the short-term endpoints reported in the literature for *P. subcapitata* and CeO_2 and TiO_2 NPs.[6,7,29,37] These concentrations exceeded those expected in the environment, but allowed a subsequent comparison of the toxicity results obtained in the present study with previous publications. The test design included three replicates at each test concentration and six control replicates. Two test sets were conducted in the presence of natural organic matter by adding 8 and 20 mg/L of SR-NOM to the growth medium of the organisms previous to the dilution of NP stock dispersions. These concentrations were representative for surface waters,[14,27] the *P. subcapitata* environments. The amounts of organic carbon in natural surface waters range from 0.5 mg C/L (sea water) to 33 mg C/L (bogs),[38] which correspond to approximately 1 to 63 mg/L SR-NOM. Independent toxicity tests were performed with Suwannee River NOM to determine its influence in the effects of the NPs studied towards algae.

Algae were grown under sterile conditions during the tests, using an orbital shaker (GFL, 3020 model) at 65 rpm and 23 ± 2 °C. Continuous illumination of 80 ± 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ (measured in the wavelength range of 400-700 nm) was provided by cool white fluorescent tubes about 30 cm distance from the position of the cultures. The light intensity was maintained within ±15% from the average over the incubation area. In

[37] Collin B, Auffan M, Johnson AC, Kaur I, Keller AA, Lazareva A, Lead JR, Ma X, Merrifield RC, Svendsen C, White JC, Unrine JM. **2014.** Environmental release, fate and ecotoxicological effects of manufactured ceria nanomaterials. *Environ Sci: Nano* 1:533-548.

[38] Thurman EM. Organic Geochemistry of Natural Waters. Chapter 1: Amount of Organic Carbon in Natural Waters. In Nijhoff M, Junk W eds, Developments in Biogeochemistry, vol. 2, Dordrecht, The Netherlands. 1985.

addition, the position of each flask in the incubator was changed every 24 h in order to compensate any lack of uniformity in the illumination system.

Growth inhibition was quantified at 24, 48 and 72 h, and tentative test endpoints were determined by calculating the concentrations bringing 10% and 50% inhibition (EC10 and EC50, respectively) as well as their associated 95% confidence limits (CL) after the 72 h exposure. These data were determined by regression analysis in Excel 2007 (Microsoft Corporation).

Chlorophyll-a extractions and fluorescence measurements

Chlorophyll-a extractions were performed to estimate the biomass concentrations of the algal cultures by means of a modified version of the fluorescence method specified in OECD-201.[39] This technique has been previously demonstrated to be useful in the assessment of the effects of CeO_2 and TiO_2 NPs on green algae.[7,40] The presence of NPs interfere with measurements of culture density normally made by optical absorbance. Extracted chlorophyll allowed the particulates and cell debris to be settled to the bottom of the tubes, whilst the chlorophyll remained in solution and was measured fluorometrically.

Samples of 1 mL from each flask containing the test cultures were extracted in a foil-wrapped screw-capped polypropylene test tube. Then, 0.1 mL of 1.5 mg/L Locust Bean Gum (Sigma-Aldrich) suspension in ultrapure water, and 4.4 ml acetone (Scharlab, HPLC grade) with $MgCO_3$, were added. The tubes were capped and inverted several times to mix, and placed in a dark cupboard at room temperature (22 ± 1°C) for 1-7 days. The samples were not exposed to bright light or air to avoid oxidative and photochemical destruction, since chlorophyll is sensitive to light and oxygen, especially when it is extracted. Homogenization of the samples was carried out to increase the extraction efficiency.

The fluorescence of the samples was determined in arbitrary units on a microplate reader (FLUOstar OPTIMA, BMG-LABTECH, Ortenberg, Germany) with an excitation wavelength of 430 nm and a measured emission wavelength of 670 nm. Measurements were performed after 24 hours extraction at room temperature and again 7 days later to check that they remained stable for that period. Fluorescence figures were corrected for background fluorescence measured on solvents mixed

[39] Mayer, P., Cuhel, R., and Nyholm N. **1997**. A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Water Res* 31:2525-2531.

[40] Cardinale BJ, Bier R, Kwan C. **2012**. Effects of TiO_2 nanoparticles on the growth and metabolism of three species of freshwater algae. *J Nanopart Res* 14:913.

with algal growth medium. The needed sub-sample volume was 350 µL in 96-well Polypropylene black microplates.

A ten-point linear calibration curve (see Figure 6.3.2) was performed to obtain the algal biomass values from fluorescence measurements. A single algal culture of 5×10^5 cells/mL was obtained and a tenfold dilution series (3×10^3 to 5×10^5 cells/mL) was prepared in 10 mL vials. Three replicates from each cell density were extracted to carry out the fluorescence measurements, and the corresponding standard curves (log cells/mL vs. log fluorescence) were represented.

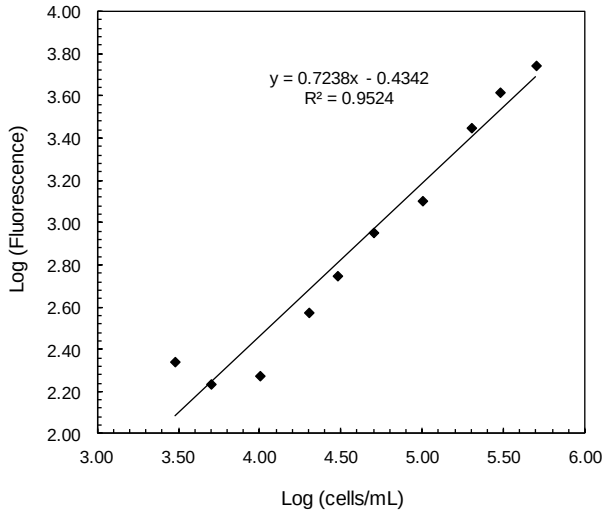

Figure 6.3.2 Calibration curve obtained from chlorophyll fluorescence in different algal concentrations (3×10^3 to 5×10^5 cells/mL). The straight lines are linear least-squares fit to the data. Excitation = 430 nm. Emission = 670 nm.

6.3.2.4. Characterization of NP dispersions

Dynamic light scattering (DLS), UV/Vis spectroscopy, and scanning electron microscopy (SEM) characterization were conducted in the NP dispersions immediately after their preparation. The dispersions with the highest exposure concentrations (160 mg/L) were characterized at the beginning and end of the tests to assess their colloidal stability and agglomeration rate as a function of time. The characterization of the dispersions at the end of the tests was preceded by a slight shaking for homogenization. Sampling only the aquatic phase would have required additional settling or centrifugation steps because sedimented or agglomerated MWCNTs were not clearly observed.

The stability of the stock dispersions and the 160 mg/L NP test dispersions was assessed by measuring the variation in calculated average zeta-sizes during the exposure period. For this purpose, Zeta-average diameter (Z_{ave}) and polydispersity

index (PDI) were obtained by DLS measurements in a Malvern Zetasizer Nano ZS instrument, considering the data generated from ten repeated measurements. In addition, zeta potentials of the test dispersions were obtained at the beginning of the tests to determine the influence of NOM in the electrophoretic mobility (EPM) of the NPs.

The DLS characterization was supported by qualitative analysis of the agglomeration during the tests, conducted in the 160 mg/L NP dispersions by measuring their total absorbance of light. UV/Vis spectroscopy is a widely used technique to analyze the stability of CeO_2 and TiO_2 NPs, given its rapidness and low cost. An UV/Vis/NIR spectrophotometer (Lambda 950, PerkinElmer) and quartz cells with 10 mm path length were used for this purpose. Calibration curves based on multi-concentration dispersions of CeO_2 and TiO_2 NPs in Milli-Q water (Figure 6.3.3) were used as a reference to perform this analysis.

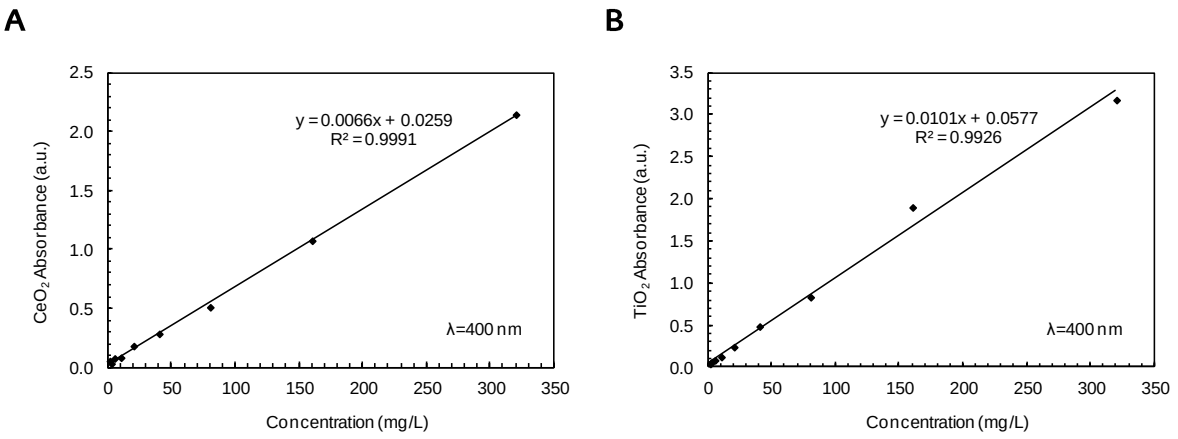

Figure 6.3.3 Calibration curves obtained from UV/Vis absorbance of CeO_2 (A) and TiO_2 (B) NPs dispersions in Milli-Q water, based on several concentrations (1.25 to 320 mg/L). The straight lines are linear least-squares fit to the data.

The selection of the wavelength was carried out considering the previously reported values for these NPs,[3,41,42] and the fact that saturation of the spectrophotometer was reached in the regions of the spectrum near their absorbance peaks (approximately 305 nm for both CeO_2 and TiO_2, Figure 6.3.4). In addition, the absorbance of organic matter (Figure 6.3.5) could have interfered with those of CeO_2 and TiO_2. The absorbance measurements were performed at 400 nm, where the absorbance of SR-

[41] Li Z, Sahle-Demessie E, Hassan AA, Sorial GA. **2011**. Transport and deposition of CeO_2 nanoparticles in water-saturated porous media. *Water Res* 45:4409-4418.

[42] Erhayem M, Sohn M. **2014**. Effect of humic acid source on humic acid adsorption onto titanium dioxide nanoparticles. *Sci Total Environ* 470-471:92-98.

NOM was negligible and did not alter those obtained for the NPs studied. Nonetheless, the measurements were carried out taking SR-NOM as background substance and subtracting their absorbance by the "autozero" function of the spectrophotometer. The almost negligible absorbance of the nutrients in the growth medium was also subtracted.

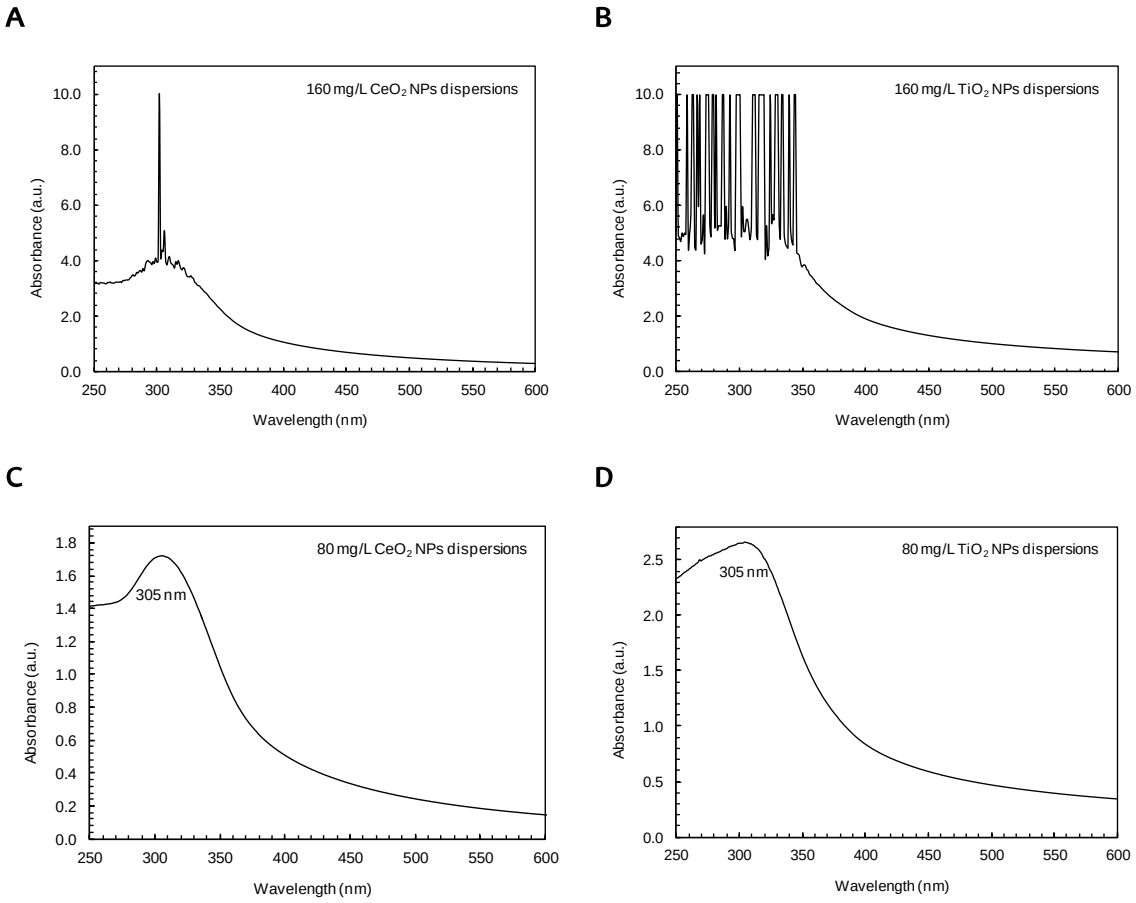

Figure 6.3.4 UV/Vis spectra of the CeO_2 and TiO_2 test dispersions. Saturation of the spectrophotometer was reached near the absorbance peaks of the 160 mg/L NPs dispersions (A, B). Therefore, they were diluted to 80 mg/L (C, D) to observe exactly these peaks (approximately at 305 nm).

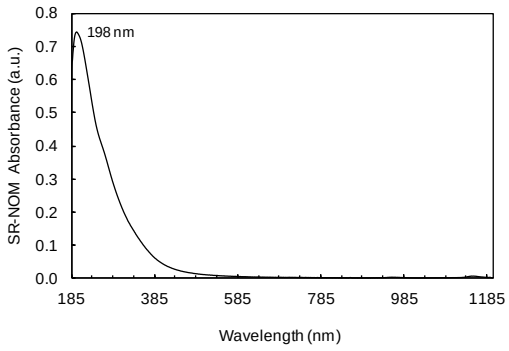

Figure 6.3.5 UV/Vis spectra of 20 mg/L SR-NOM in Milli-Q water.

Furthermore, the results obtained in the previous characterization were complemented by SEM imaging, using a ZEISS apparatus (ULTRA PLUS model). The SEM samples were prepared by a drying process for 24 h under ambient temperature.

6.3.3. Results and Discussion

6.3.3.1. Characterization by DLS

The results of the DLS measurements performed in the stock dispersions and the 160 mg/L NP test dispersions are shown in Tables 6.3.3 and 6.3.4. Size distribution and zeta potential graphs are provided in Figures 6.3.6 to 6.3.15. The 8 and 20 mg/L SR-NOM dispersions were labelled as SRNOM-8 and SRNOM-20, respectively.

Table 6.3.3 Zeta-average diameters (Z_{ave}) and polydispersity indices (PDI) of the stock dispersions and 160 mg/L NP test dispersions at the beginning and end of the tests.

	CeO_2 NPs				TiO_2 NPs			
	$Z_{ave,mean}$ (nm)	SD	PDI_{mean}	SD	$Z_{ave,mean}$ (nm)	SD	PDI_{mean}	SD
Stock dispersions	196.8	9.6	0.345	0.056	151.6	1.0	0.334	0.008
Test dispersions-0h	2557.7	613.8	0.301	0.040	2389.3	376.4	0.244	0.040
Test dispersions-72h	1939.7	307.7	0.279	0.017	2697.0	368.6	0.203	0.042
Test dispersions + SRNOM-8-0h	176.4	4.5	0.234	0.007	175.3	8.9	0.259	0.056
Test dispersions + SRNOM-8-72h	235.3	17.1	0.219	0.015	178.7	10.9	0.270	0.023
Test dispersions + SRNOM-20-0h	167.9	7.1	0.215	0.025	152.6	1.9	0.245	0.007
Test dispersions + SRNOM-20-72h	259.4	8.7	0.235	0.012	173.8	29.2	0.316	0.056

SD = standard deviation of measurements corresponding to three test replicates.

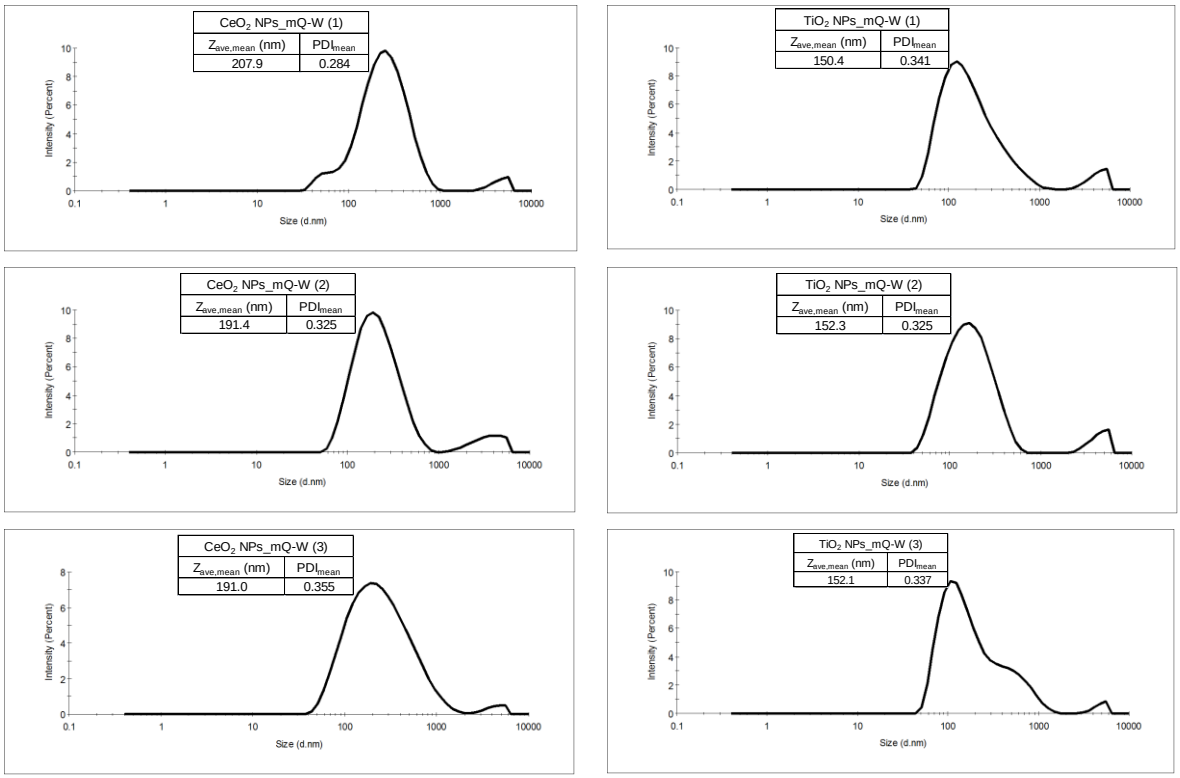

Figure 6.3.6 Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in stock dispersions. Measurements correspond to three test replicates.

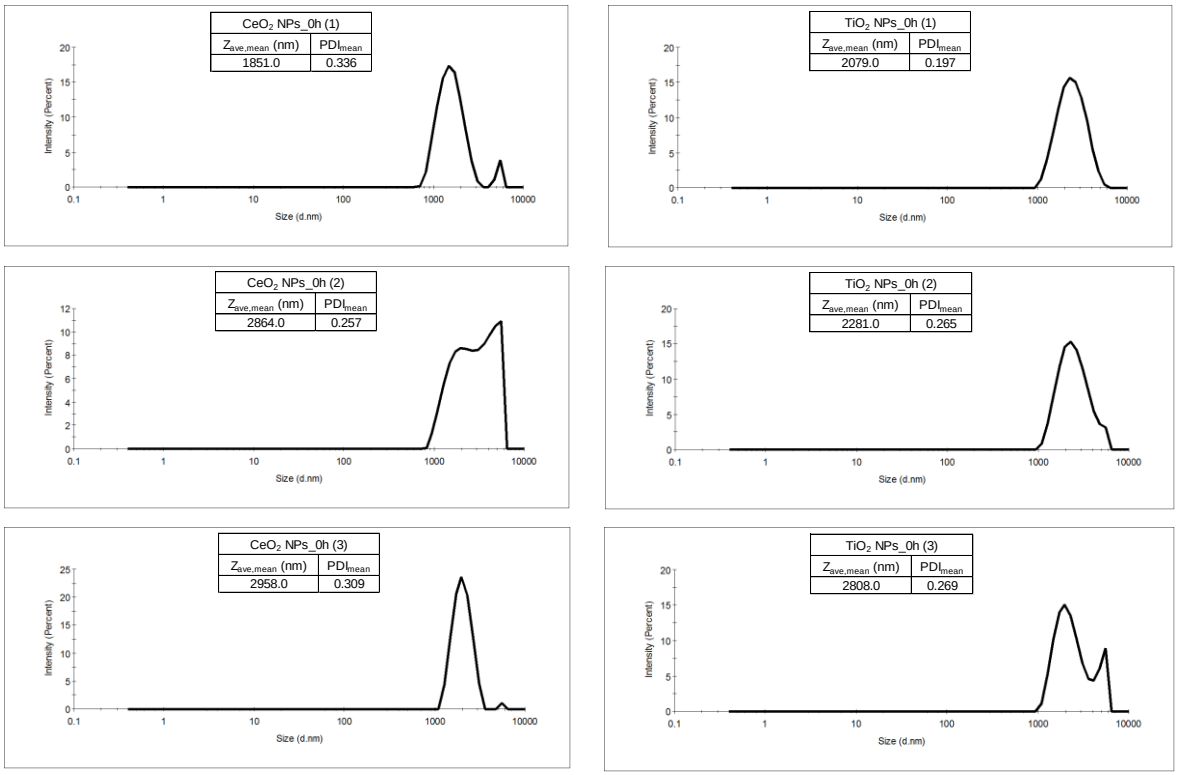

Figure 6.3.7 Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions in the absence of SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

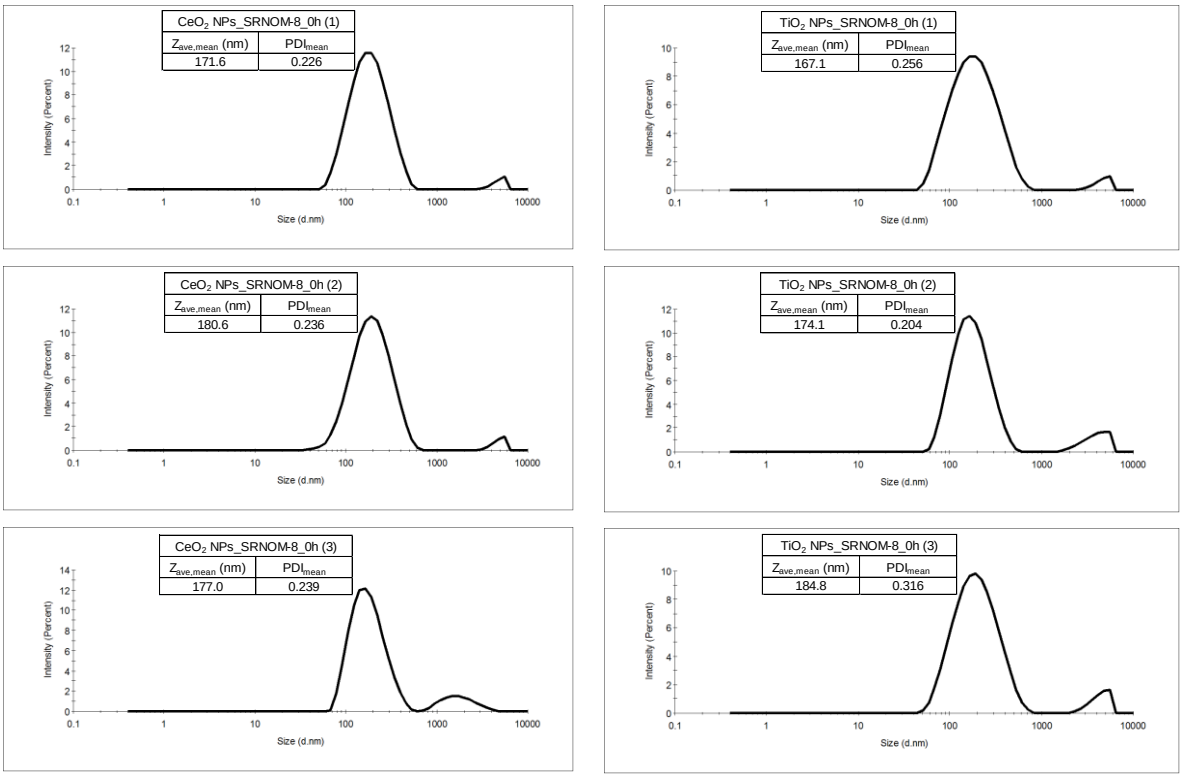

Figure 6.3.8 Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 8 mg/L SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

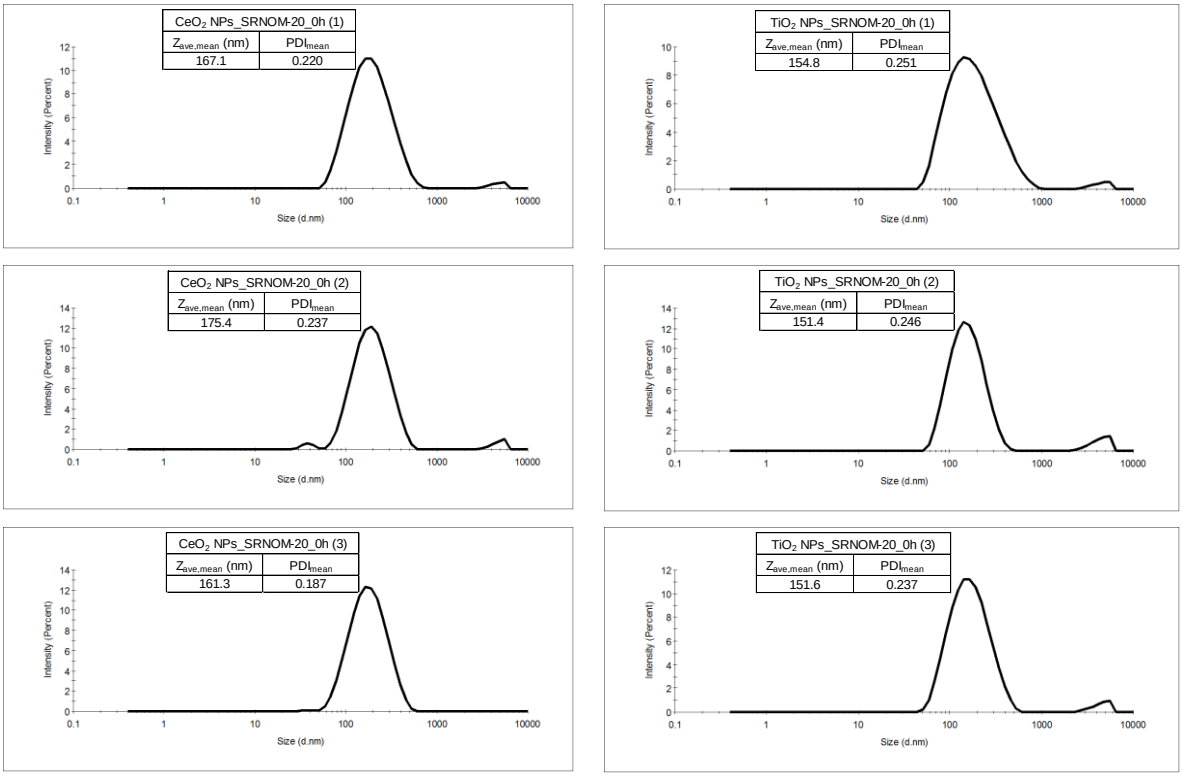

Figure 6.3.9 Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 20 mg/L SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

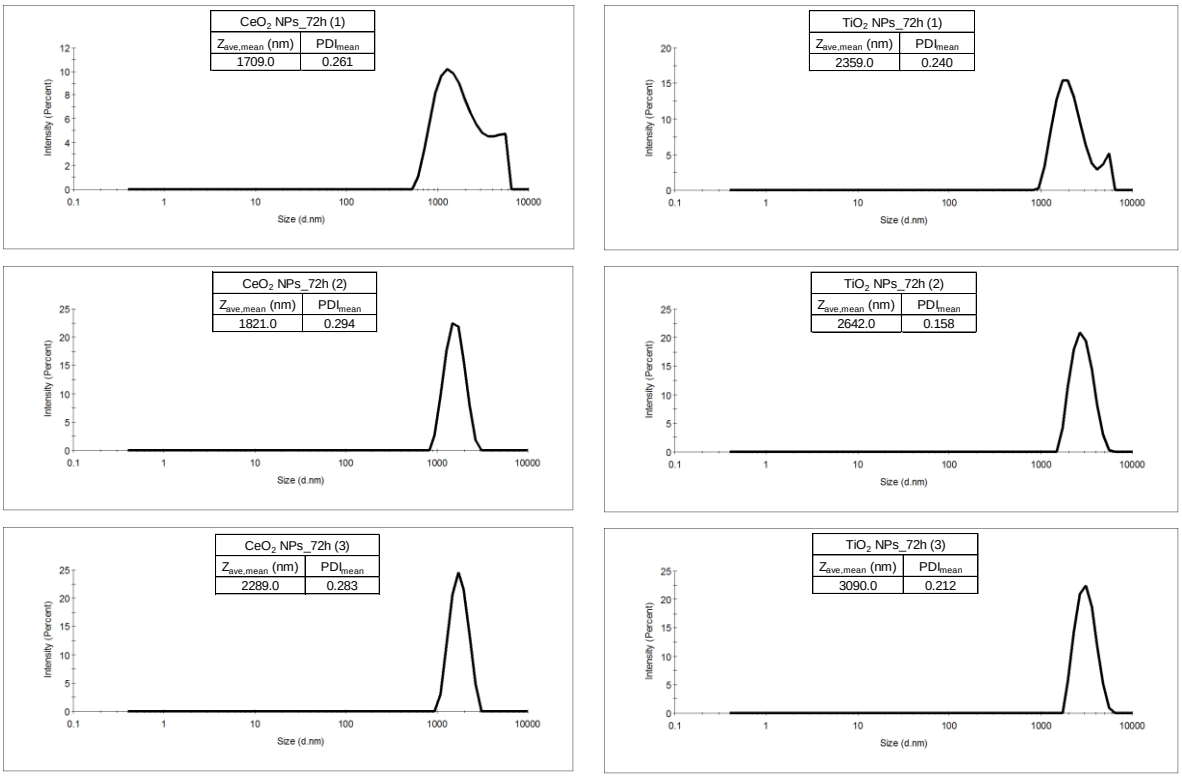

Figure 6.3.10 Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions in the absence of SR-NOM at the end of the tests. Measurements correspond to three test replicates.

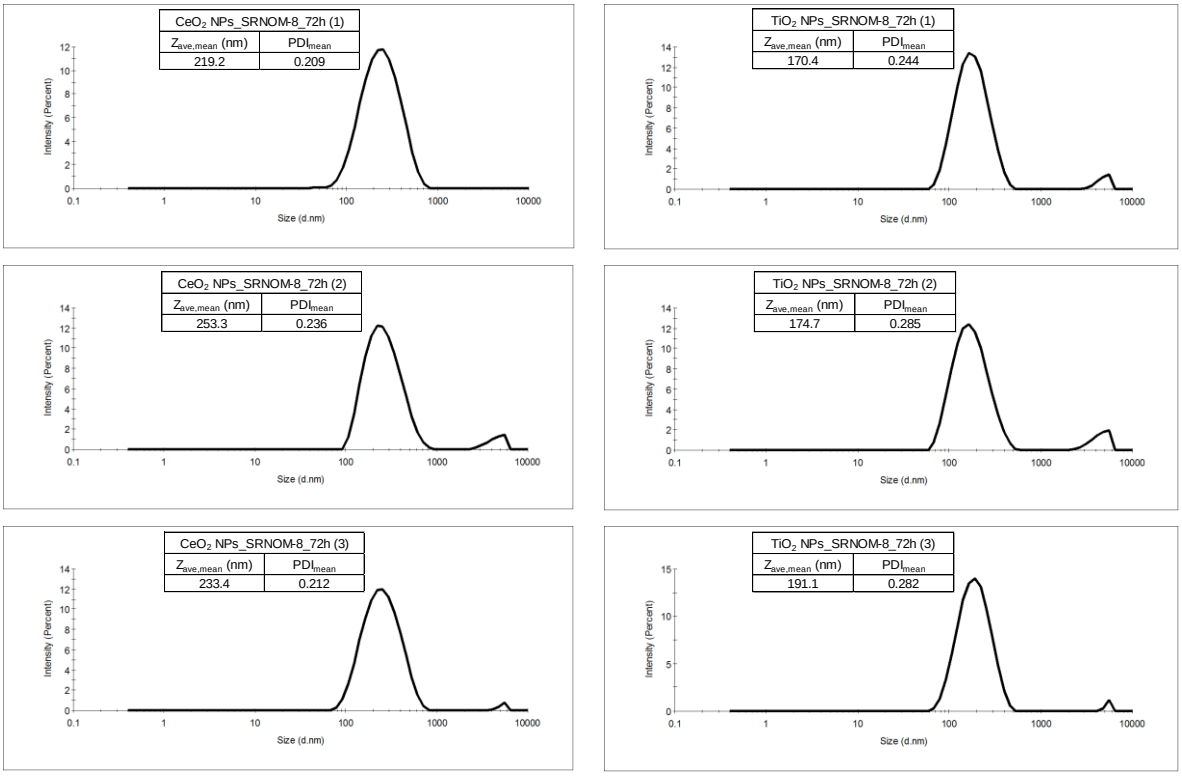

Figure 6.3.11 Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 8 mg/L SR-NOM at the end of the tests. Measurements correspond to three test replicates.

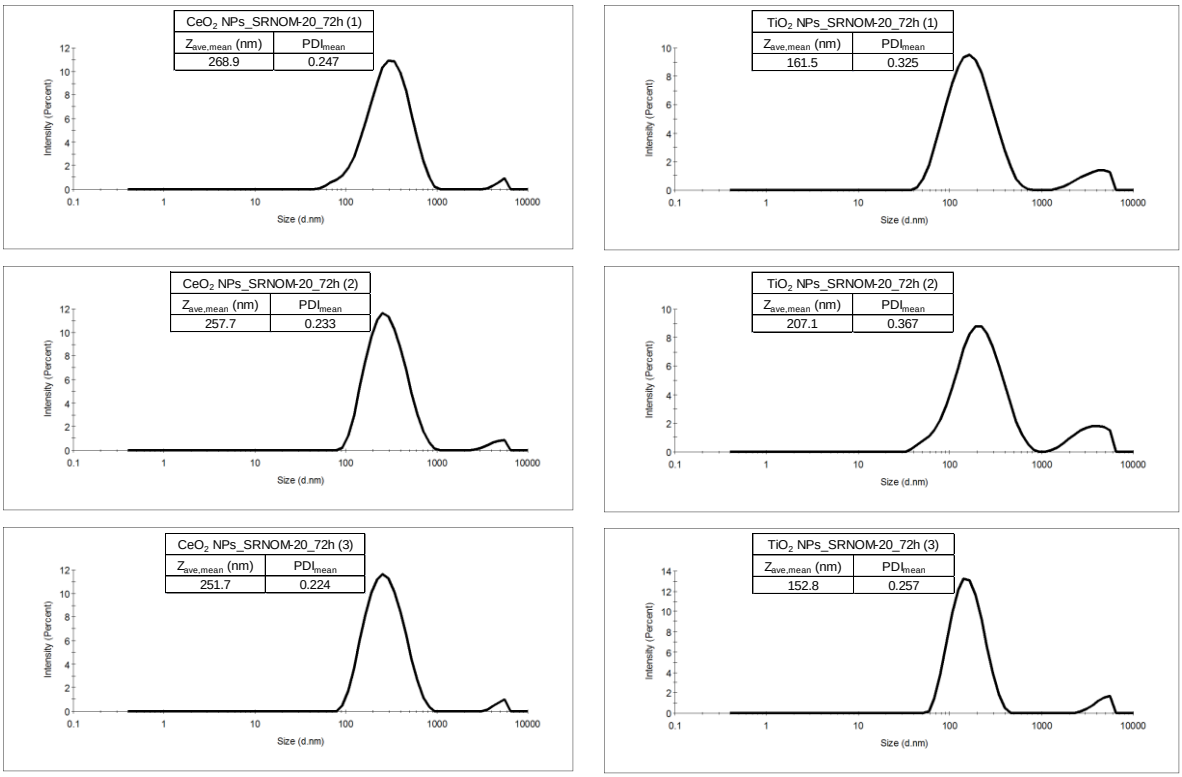

Figure 6.3.12 Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 20 mg/L SR-NOM at the end of the tests. Measurements correspond to three test replicates.

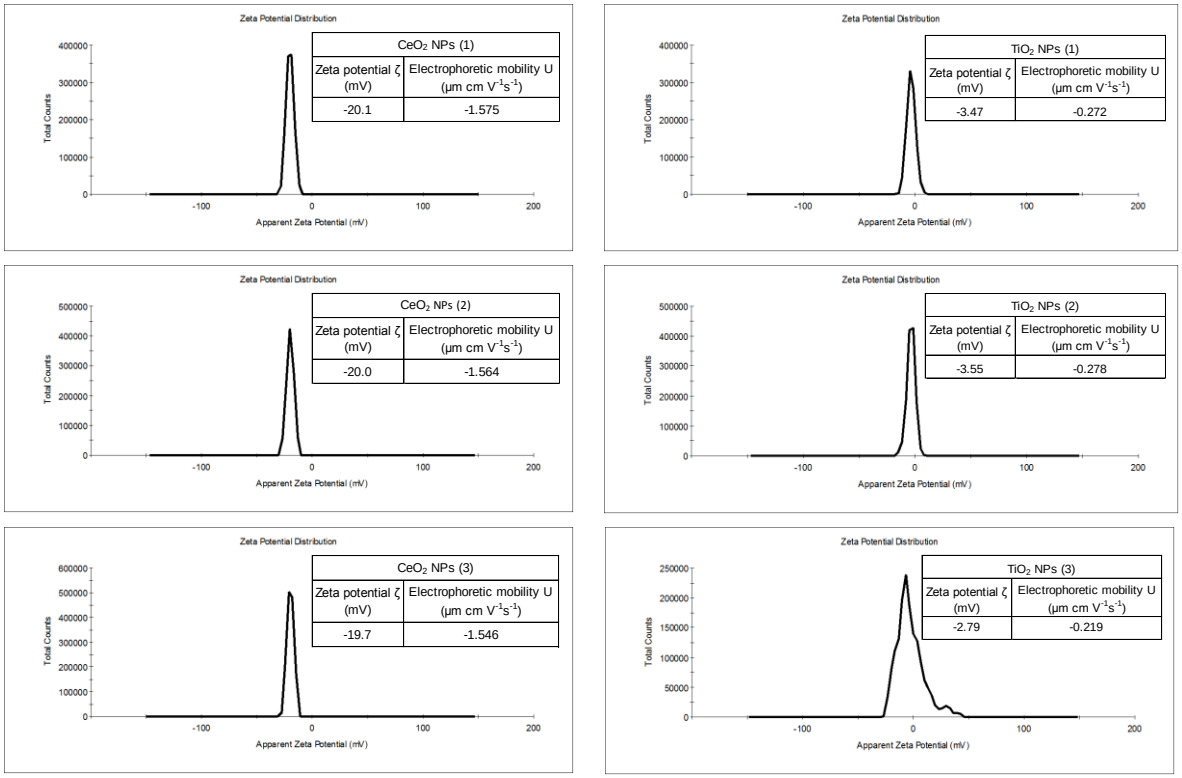

Figure 6.3.13 Zeta potential distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions in the absence of SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

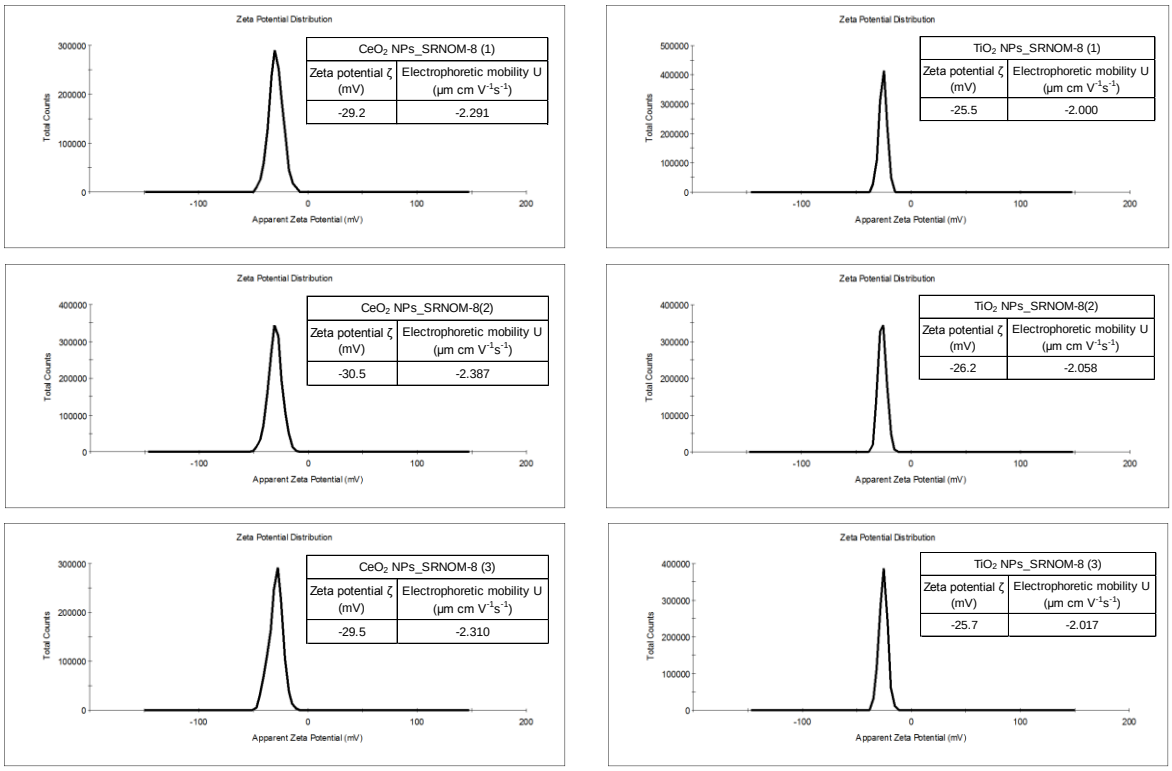

Figure 6.3.14 Zeta potential distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 8 mg/L SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

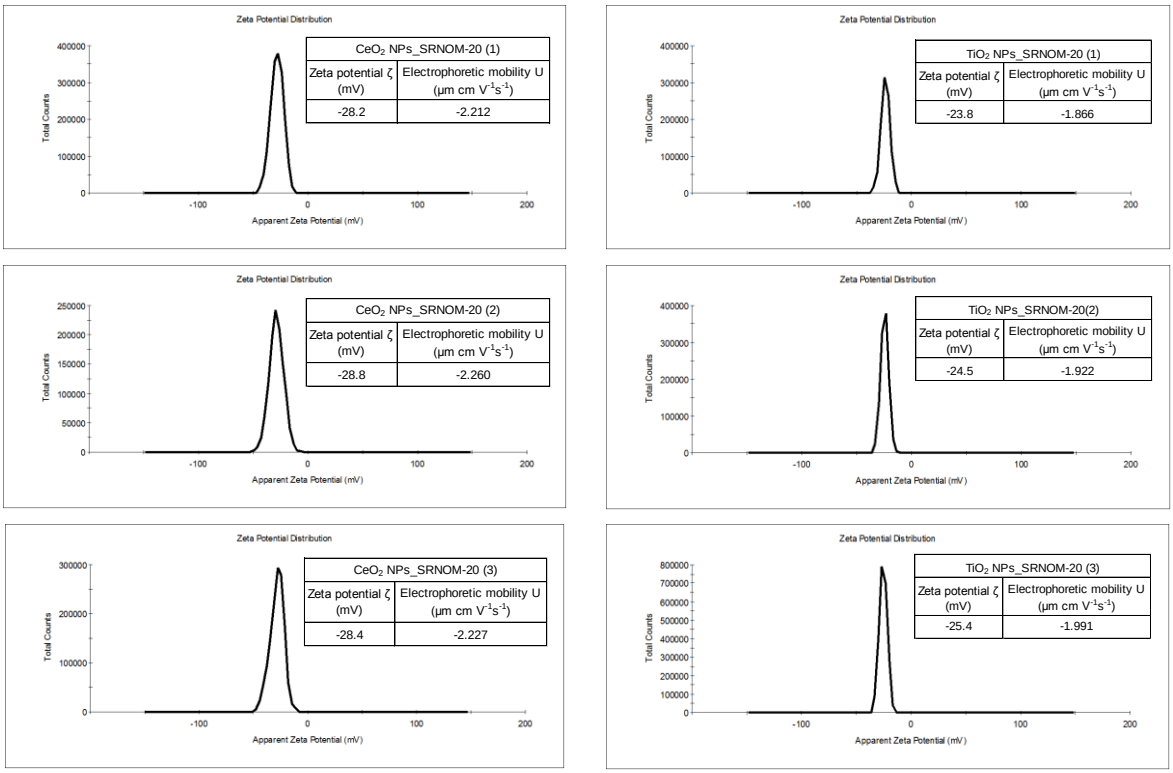

Figure 6.3.15 Zeta potential distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 20 mg/L SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

The agglomerate sizes in stock dispersions were consistent with their nominal particle sizes in the primary characterization, and for both CeO_2 and TiO_2, Z_{ave} was approximately six times greater than the primary NP size (Tables 6.3.1 and 6.3.3). It was also experimentally observed that the dilution of the stock dispersions into the algal growth medium substantially increased the Z_{ave} of CeO_2 and TiO_2 NPs to a 2-3 µm range. This behavior, caused by the nutrients present in the medium, was significantly altered in the presence of both 8 mg/L and 20 mg/L SR-NOM, since the NPs maintained approximately the same agglomerate sizes obtained in stock dispersions even at the end of the exposure period (Figures 6.3.16A and 6.3.16B). This outcome demonstrated the increased stability provided by SR-NOM to CeO_2 and TiO_2 dispersions, which can be explained on the basis of the strong adsorption of organic matter to metal oxide nanoparticles.[14,15] Increasing SR-NOM concentrations resulted in a slight decrease of Z_{ave} in most cases, but these variations were negligible in the range of the agglomerate sizes obtained.

Concerning the PDI, the low values obtained for the stock dispersions and over the duration of the tests (between 0.203 and 0.345) indicated that DLS was a suitable technique to determine the stability of the NPs in this study. High PDI is considered a limiting factor for the use of DLS in particle size characterization.[1,31] The test dispersions showed lower polydispersity than the stock dispersions in all cases (Figures 6.3.16C and 6.3.16D). These narrower size distributions might be a result of dilution itself from 2560 mg/L to 160 mg/L, although the presence of the nutrient salts in the algae growth medium could also influence polydispersity. The presence of nutrient salts, which determine the ionic strength of the aqueous medium, and the MNM concentrations have been reported to affect their agglomeration kinetics and stability.[3,10,18,30] Nonetheless, the PDI values in the presence and absence of SR-NOM were different for CeO_2 and TiO_2 NPs. A decreasing trend was observed in the polydispersity of the CeO_2 test dispersions after adding SR-NOM, whilst TiO_2 showed the opposite behavior. This fact might constitute an indicator of the different effects of SR-NOM on the agglomeration kinetics and ecotoxicity of different NPs, mentioned in the *Introduction* section. The higher values observed for PDI of TiO_2 dispersions in the presence of SR-NOM were probably caused by the exopolymeric substances excreted by algae to mitigate the stress induced, cited as a contributor to

agglomeration.[43] These exudates are considered a much bigger problem for nanomaterials experiments compared to traditional chemicals,[44] and might be more abundant in TiO_2 NP dispersions, considering the high algal growth rates in the presence of SR-NOM (see *Algae ecotoxicity studies* subsection). This behavior can also be related to the critical coagulation concentration of TiO_2 NPs, which have shown higher sedimentation rates than CeO_2 NPs in natural aqueous media.[3]

Regarding the variation in calculated DLS parameters as a function of time, a slight increase in the agglomerate sizes and PDI after 72 h of exposure was observed in the presence of SR-NOM in most cases. It was probably because the alterations in the algal growth introduced by the organic matter (see *Algae ecotoxicity studies* subsection) or the above mentioned presence of exudates might contribute to agglomeration. In the tests performed in the absence of organic matter, PDI showed the opposite trend and decreased at the end of the exposure period. Z_{ave} did not show a clear trend over the test duration and presented considerable standard deviation. This fact indicated that SR-NOM not only improved the stability of the dispersions, but also contributed to a better homogeneity of the DLS results over time.

[43] Hartmann NB, Von der Kammer F, Hofmann T, Baalousha M, Ottofuelling S, Baun A. **2010**. Algal testing of titanium dioxide nanoparticles-testing considerations, inhibitory effects and modification of cadmium bioavailability. *Toxicology* 269:190-197.

[44] Handy RD, van den Brink N, Chappell M, et al. **2012**. Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? *Ecotoxicology* 21:933-972.

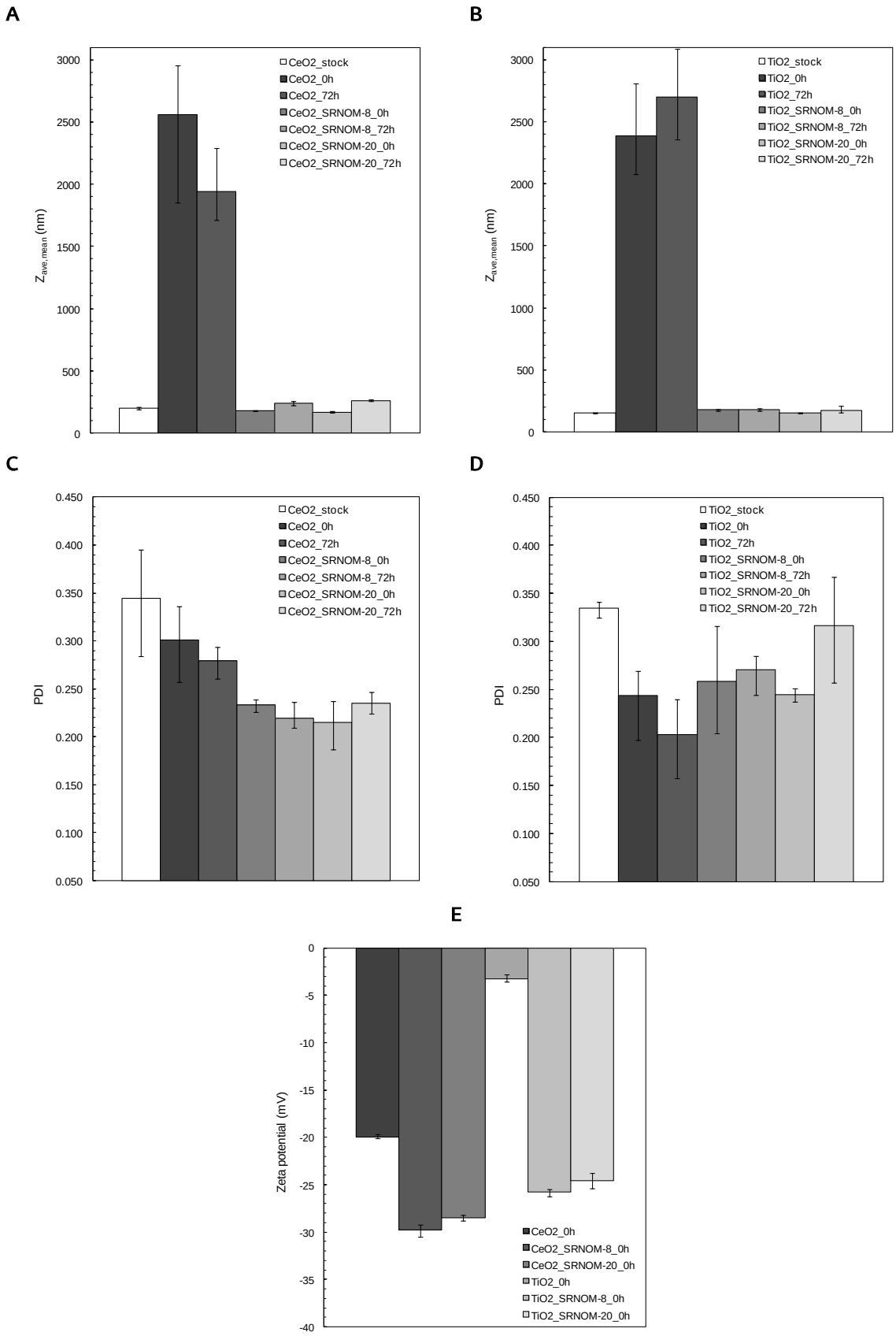

Figure 6.3.16 Histogram comparisons of Z_{ave} size (A,B), PDI (C,D) and zeta potential (E) of the stock dispersions and 160 mg/L NP test dispersions at the beginning and end of the tests. Error bars represent standard deviation (n=3).

The DLS results obtained in the present study were similar to those previously reported in the literature. Zeta-average diameters and PDI of CeO_2 and TiO_2 NPs were influenced mainly by their physico-chemical properties, the methods and growth medium used to prepare the dispersions, and the type of NOM added. CeO_2 NPs, with primary particle size of 20 nm studied by Quik et al.,[14] reduced their Z_{ave} in *P. subcapitata* growth medium from 417 nm to 248 nm after adding SR-NOM. Cupi et al.[27] also analyzed the SR-NOM effect on the agglomerate sizes of 25-nm-diameter TiO_2 NPs, and observed a decrease in Z_{ave} from 1325 nm to 101 nm (maximum and minimum values obtained, respectively) in *D. magna* medium. Nevertheless, these studies did not provide the amount of acoustic energy delivered to the NPs during the preparation of dispersions, which determines the hydrodynamic particle size-distributions. The sonicator's power setting value does not indicate itself accurately the effective acoustic power.[34] The standardization approach of the present study included the calculation of the energy delivered to the NPs during the preparation of dispersions (see *Materials and Methods* section) to allow a fully reproducible method. With respect to PDI, Cupi et al.[27] obtained values of up to 0.88 and 0.39 in the absence and presence of SR-NOM, respectively (which were not as low as the values observed in the present study), and Quik et al.[14] did not provide them. This fact suggests a better optimization of the energy delivered to the NPs in the dispersion process.

Table 6.3.4 Zeta potential and electrophoretic mobility of the 160 mg/L NP test dispersions at the beginning of the tests.

	CeO_2 NPs				TiO_2 NPs			
	Zeta potential ζ (mV)	SD	EPM U (μm cm $V^{-1}s^{-1}$)	SD	Zeta potential ζ (mV)	SD	EPM U (μm cm $V^{-1}s^{-1}$)	SD
Test dispersions	-19.9	0.2	-1.562	0.015	-3.3	0.4	-0.257	0.033
Test dispersions + SRNOM-8	-29.7	0.7	-2.329	0.051	-25.8	0.4	-2.025	0.030
Test dispersions + SRNOM-20	-28.5	0.3	-2.233	0.025	-24.6	0.8	-1.926	0.063

SD = standard deviation of measurements corresponding to three test replicates.

EPM = Electrophoretic mobility

The electrophoretic mobility and the zeta potential values obtained (Table 6.3.4 and Figure 6.3.16E) showed that CeO_2 NPs exhibited higher stability than TiO_2 NPs in algal growth medium alone. The reason underlying might be the high tendency of CeO_2 NPs to adsorb phosphate ions dissolved in the algal growth medium.[7,45] The addition of SR-NOM resulted in even more negative zeta potentials for both CeO_2 and TiO_2 test dispersions. SR-NOM concentration did not influence EPM significantly, considering the negligible differences and the standard deviations observed in zeta potentials of 8 mg/L and 20 mg/L SR-NOM samples. The dramatic enhancement of the TiO_2 NPs EPM after adding SR-NOM was consistent with the more pronounced reduction in its Z_{ave} compared to that of CeO_2 NPs (see Figures 6.3.16A and B). This behavior could be related to the greater particle surface area of TiO_2 NPs, which might lead to an increase of the amount of SR-NOM adsorbed with respect to CeO_2 NPs (see Table 6.3.1). Additional SEM characterization was conducted on stock dispersions of NPs to further analyze this phenomenon at the nanoparticle level (Figure 6.3.17).

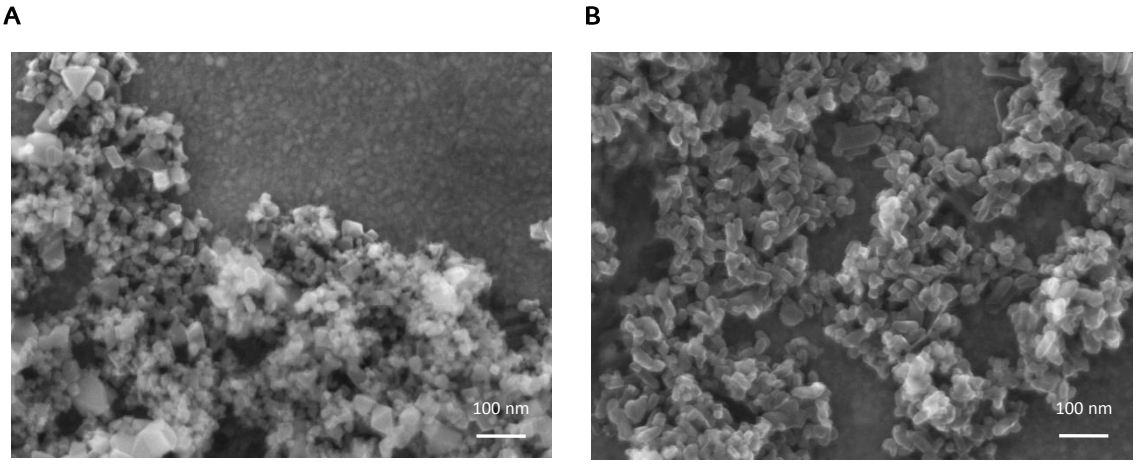

Figure 6.3.17 Field-emission scanning electron microscopic images obtained with a JEOL apparatus (JSM-7000F model) of: (A) CeO_2 NPs stock dispersions and (B) TiO_2 NPs stock dispersions.

The considerable variability observed in CeO_2 primary particle sizes influenced their low particle surface area compared to that of TiO_2 NPs. Furthermore, the round or elongated shape of TiO_2 NPs probably promoted the adsorption of SR-NOM, whilst the polyhedral morphology of CeO_2 NPs hindered their interaction with SR-NOM because of a directional adsorption mechanism. Electrophoretic mobility of CeO_2

[45] Van Hoecke K, Quik JTK, Mankiewicz-Boczek J, De Schamphelaere KAC, Elsaesser A, Van der Meeren P, et al. **2009**. Fate and effects of CeO_2 nanoparticles in aquatic ecotoxicity tests. *Environ Sci Technol* 43:4537-4546.

and TiO_2 NP dispersions tended to similar values in the presence of SR-NOM. A previous study on this issue[3] reported the same behavior for EPM of CeO_2 and TiO_2 NPs in various river and groundwaters. This fact suggests that SR-NOM might be a representative sample of what is found in many different ecosystems, and hence fulfill the need for standardization of ecotoxicity tests. The zeta potential values reported in the literature for CeO_2 NPs in the presence of SR-NOM,[14] and for TiO_2 NPs in the presence of humic acids from Suwannee River[23,25] were also in accord with the ranges observed in the present study.

6.3.3.2. Characterization by UV/Vis spectroscopy

Generally, the UV/Vis characterization supported the results obtained with DLS measurements. The more pronounced changes in the electrophoretic mobility and Z_{ave} of TiO_2 NPs compared to that of CeO_2 NPs in the presence of SR-NOM were in good agreement with their greater absorbance after adding organic matter (see Figure 6.3.18).

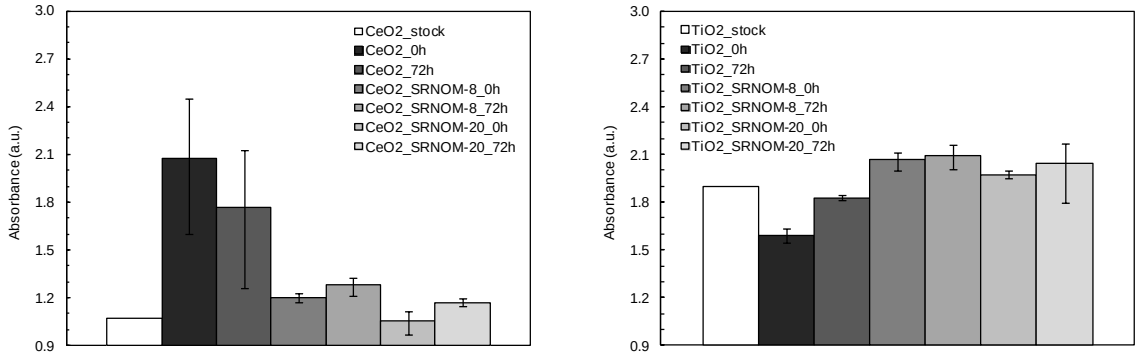

Figure 6.3.18 Histogram comparisons of UV/Vis absorbances of the 160 mg/L NP dispersions performed at 400 nm. Error bars represent standard deviation (n=3).

The fact that low Z_{ave} results in higher UV/Vis absorbances is generally accepted. Large agglomerates are more prone to destabilization and sedimentation, which result in lower UV/Vis absorbance.[3,31,42] The increase observed in the absorbance values of TiO_2 dispersions might be caused by their smaller agglomerate sizes with respect to those of CeO_2 dispersions, apart from their crystalline structure, which also determines their UV/Vis spectra (Figure 6.3.4). In the case of CeO_2, the test dispersions prepared in the presence of SR-NOM showed lower optical absorbance with respect to those prepared in growth medium alone. The considerable standard deviation obtained in the absence of SR-NOM constitutes an indicator of the instability of these dispersions and might explain these anomalous results. Their

limited reliability was also supported by the similar absorbance of CeO_2 test dispersions in the presence of organic matter and that of 160 mg/L calibration standards. SR-NOM concentrations did not appear to substantially impact the absorbance values of CeO_2 and TiO_2 dispersions, considering the standard deviations obtained.

With regard to the slight increase obtained in calculated Z_{ave} and PDI over the exposure period in the presence of SR-NOM, the absorbance remarkably also revealed higher values at 72 h, despite their standard deviation values. It was probably caused by the previously commented presence of exopolymeric exudates, which might absorb in the wavelength range selected for the UV/Vis measurements, thus interfering and increasing the absorbance measured in CeO_2 and TiO_2 dispersions.

The UV/Vis analysis was performed on a qualitative basis with the purpose of supporting the data obtained in the characterization by DLS. Therefore, it was difficult to directly compare the absorbance results with those reported in previous research.[3,41,42] which have often conducted quantitative analysis to calculate the variations in normalized NP concentrations as a function of time. Nonetheless, the absorption values found by Keller et al.[3] for CeO_2 NP dispersions were lower than that of TiO_2 NP dispersions, which is in accordance with the results obtained in the present study.

6.3.3.3. Characterization by scanning electron microscopy (SEM)

Electron microscopy has been used in previous studies to illustrate the differences in the agglomerate sizes of CeO_2 and TiO_2 NPs dispersed under various methods in algal media.[16,46,47] The test dispersions prepared in the present study exhibited quite low polydispersity in the DLS characterization carried out. However, different agglomerate sizes were still found and it is well-known that SEM images permits only the visualization of a tiny area of the dispersions. Moreover, the preparation of SEM samples involved changes in the ultimate disposition of the nanoparticles studied. During their drying process, NOM and growth medium substances could have crystallized, and CeO_2 and TiO_2 NPs possibly formed larger agglomerates. Therefore,

[46] Manier N, Bado-Nilles A, Delalain P, Aguerre-Chariol O, Pandard P. **2013**. Ecotoxicity of non-aged and aged CeO_2 nanomaterials towards freshwater microalgae. *Environ Pollut* 180:63-70.
[47] Lin D, Ji J, Long Z, Yang K, Wu F. **2012**. The influence of dissolved and surface-bound humic acid on the toxicity of TiO_2 nanoparticles to Chlorella sp. *Water Res* 46:4477-4487.

this disposition was not completely comparable to their state in dispersion. In spite of these uncertainties, the overall findings and insights obtained by the previous characterization were supported by SEM.

The imaging conducted (Figures 6.3.19 and 6.3.20) supported the previously mentioned fact that SR-NOM reduced the Z_{ave}, thus increasing the stability of the test dispersions. Although variations in Z_{ave} as a function of SR-NOM concentration were not substantial, increasing organic matter concentrations resulted in decreasing agglomerate sizes in most cases (Figures 6.3.19E, 6.3.19F, 6.3.20E and 6.3.20F). In addition, SEM images contributed to observe the greater stability of TiO_2 dispersions provided by SR-NOM with respect to that of CeO_2 dispersions. For instance, the reduction in the agglomerate sizes of CeO_2 NPs shown in Figure 6.3.19 was less significant than that shown by TiO_2 NPs in Figure 6.3.20.

The increase in the Z_{ave} after 72 h of exposure, observed mainly in CeO_2 dispersions in the presence of 20 mg/L SR-NOM was illustrated in Figures 6.3.19E and 6.3.19F. As already mentioned, in the absence of SR-NOM, DLS measurements did not clearly exhibit the same trend for the test dispersions, because of considerable standard deviation. Nevertheless, the SEM imaging was useful to observe that larger agglomerates were also found after the exposure period in this case (Figures 6.3.19A, 6.3.19B, 6.3.20A and 6.3.20B). It was reasonable, taking into account that nutrient salts in the algal growth medium and the exopolymeric exudates excreted by alga during the tests contributed to agglomeration.

The influence of SR-NOM in the variations of the PDI observed for CeO_2 and TiO_2 dispersions was also shown by the SEM characterization. TiO_2 test dispersions exhibited higher PDI with increasing concentrations of SR-NOM, illustrated by the greater variability in the size distributions in Figures 6.3.20E and 6.3.20F. The opposite behavior was shown by CeO_2 test dispersions, with higher PDI in the absence of SR-NOM, observed in Figures 6.3.19A and 6.3.19B.

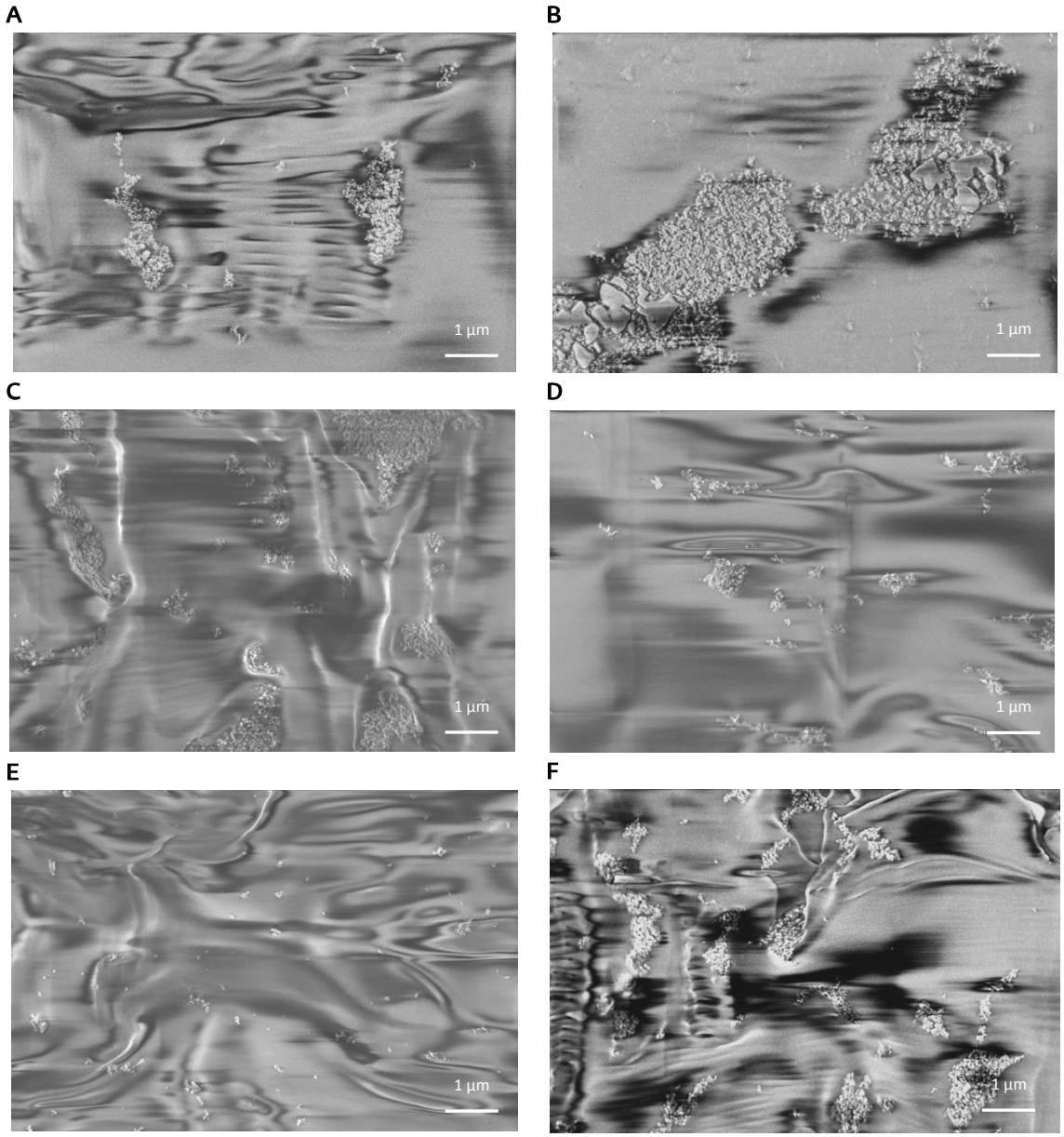

Figure 6.3.19 SEM images of the 160 mg/L NP dispersions: (A) CeO_2 _0h, (B) CeO_2 _72h, (C) CeO_2 _SRNOM-8_0h, (D) CeO_2 _SRNOM-8_72h, (E) CeO_2 _SRNOM-20_0h, (F) CeO_2 _SRNOM-20_72h.

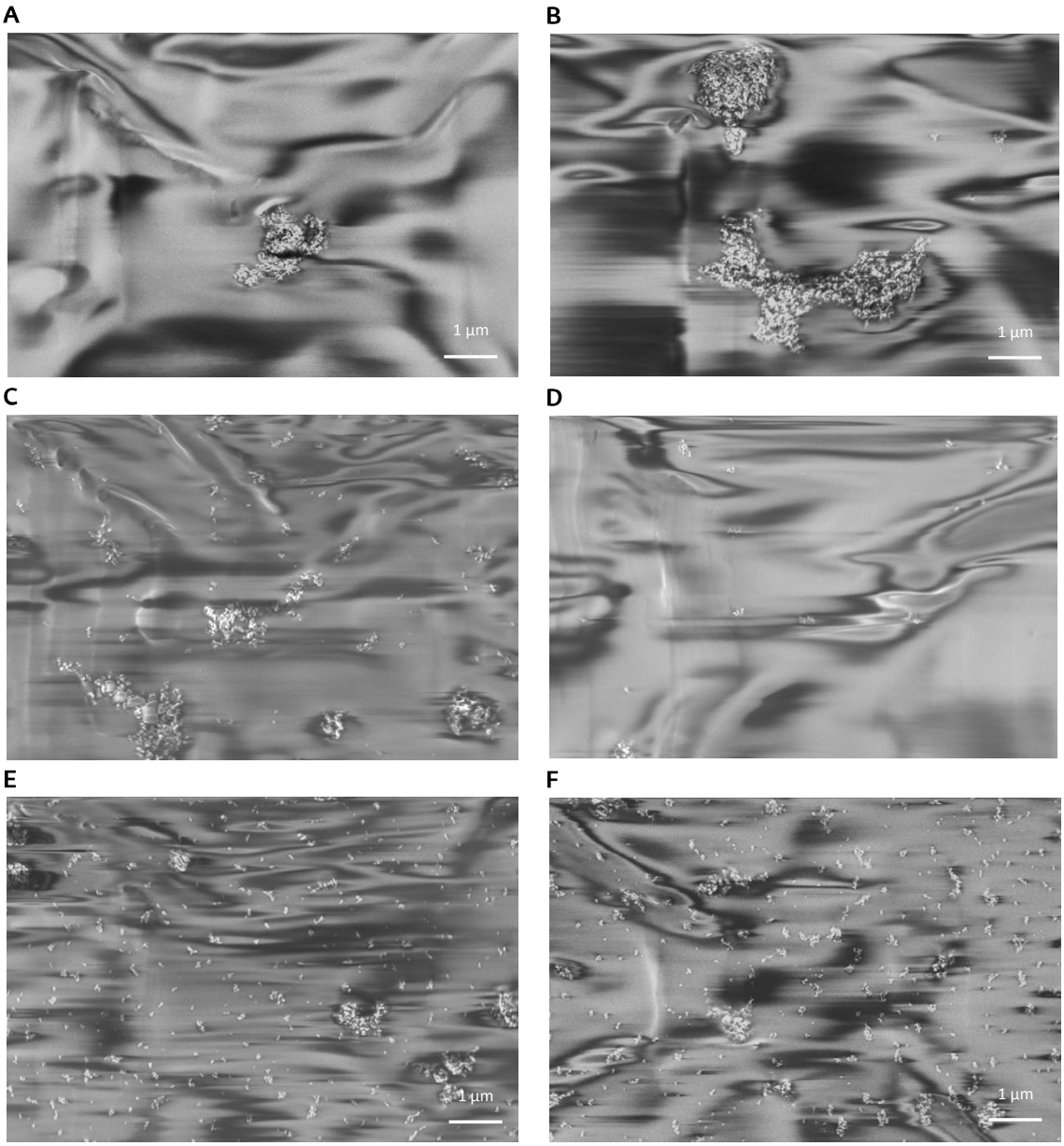

Figure 6.3.20 SEM images of the 160 mg/L NP dispersions: (A) TiO_2_0h, (B) TiO_2_72h, (C) TiO_2 _SRNOM-8_0h, (D) TiO_2_SRNOM-8_72h, (E) TiO_2_SRNOM-20_0h, (F) TiO_2_SRNOM-20_72h.

6.3.3.4. Algae ecotoxicity studies

CeO_2 and TiO_2 NPs in the absence of SR-NOM showed considerable adverse effects even at the lowest concentrations tested (Table 6.3.5 and Figure 6.3.21). Flocculation and clustering of NPs around *P. subcapitata* cells were observed in these dispersions (Figure 6.3.22), which have been previously proposed to cause artifacts in toxicity tests by a local nutrient depletion and/or shading at the cellular level.[45] The 72 h-EC50 value obtained for pristine CeO_2 NPs and *P. subcapitata* in the absence of SR-NOM in the present study (1.24 mg/L) was consistent with the variable toxic effects

reported by Manier et al. (4.1-6.2 mg/L),[46] Rodea-Palomares et al. (2.4-29.6 mg/L),[48] and Van Hoecke et al. (10.2-19.1 mg/L).[45] In the case of TiO_2 NPs, Hartmann et al.[43] found EC50 values of 71.1-241 mg/L, and Menard et al.[6] obtained values as low as 5.83 mg/L. The TiO_2 NPs analyzed in the present study showed an EC50 value of 0.27 mg/L, quite lower than those proposed in the literature, indicating even greater variability than in the case of CeO_2 NPs. Although the lack of stability of the dispersions in the absence of SR-NOM might be a determining factor in the reproducibility of the test results, the intrinsic physicochemical properties of CeO_2 and TiO_2 NPs also probably played an important role in their variable adverse effects. For instance, toxicity of CeO_2 NPs towards *P. subcapitata* has been found to increase with decreasing nominal particle size,[45] and Booth et al.[7] obtained EC50 values of 0.024 mg/L for CeO_2 NPs with sizes between 4 and 10 nm.

Table 6.3.5 Calculated 50% effective concentration (EC50) and 10% effective concentration (EC10) of NP dispersions (mg/L) to *Pseudokirchneriella subcapitata* during 72 h, and lower and upper 95% confidence intervals (CL) from the statistical analysis (n=3).

Test substance	EC50 (95% CL)	EC10 (95% CL)
CeO_2 NPs	1.24 (1.07-1.43)	-
TiO_2 NPs	0.27 (0.15-0.48)	-
SR-NOM	No inhibition (increase in the growth rate)	
CeO_2 NPs + SRNOM-8	31.9 (10.2-99.5)	16.2 (4.8-54.4)
TiO_2 NPs + SRNOM-8	No inhibition (increase in the growth rate)	
CeO_2 NPs + SRNOM-20	74.3 (18.0-305.9)	38.0 (8.4-171.6)
TiO_2 NPs + SRNOM-20	No inhibition (increase in the growth rate)	

Note: The pH values at the end of the tests decreased slightly from the initial 8.2-8.3 to average values of 7.8. In the case of metals and compounds that partly ionize at a pH around the test pH, OECD Guideline 201 requires a pH drift of less than 0.5 to obtain reproducible and well defined results. Thus, the pHs were kept in the range of the validity criteria during the exposure period. The biomass in the control cultures increased exponentially by a factor corresponding to specific growth rates of 0.9 day^{-1}. This value was lower than the specified by the OECD Guideline 201, but otherwise acceptable, taking into account that one of the nutrients in the algal growth medium was removed to avoid binding on metal ions.

[48] Rodea-Palomares I, Boltes K, Fernández-Piñas F, Leganés F, García-Calvo E, Santiago J, Rosal R. **2011**. Physicochemical characterization and ecotoxicological assessment of CeO_2 nanoparticles using two aquatic microorganisms. *Toxicol Sci* 119:135-145.

The different effective concentration values observed between CeO_2 and TiO_2 NPs were influenced by their physicochemical features, which led to specific ecotoxicity mechanisms. Reactive oxygen species (ROS) generated by CeO_2 NPs were reported to produce a loss of the lipid peroxidation recovery and radical scavenging activity of *P. subcapitata* cells during 72 h.[7] Hartmann et al.[43] proposed ecotoxicity mechanisms of TiO_2 NPs towards algae such as ROS generation, adhesion of NPs to algal cells and physical disruption of the cell membranes. The TiO_2 NPs tested in the current study showed higher content of impurities than that of CeO_2 NPs (Table 6.3.1), which might have also determine the lower EC50 values obtained in the absence of SR-NOM.[43,44]

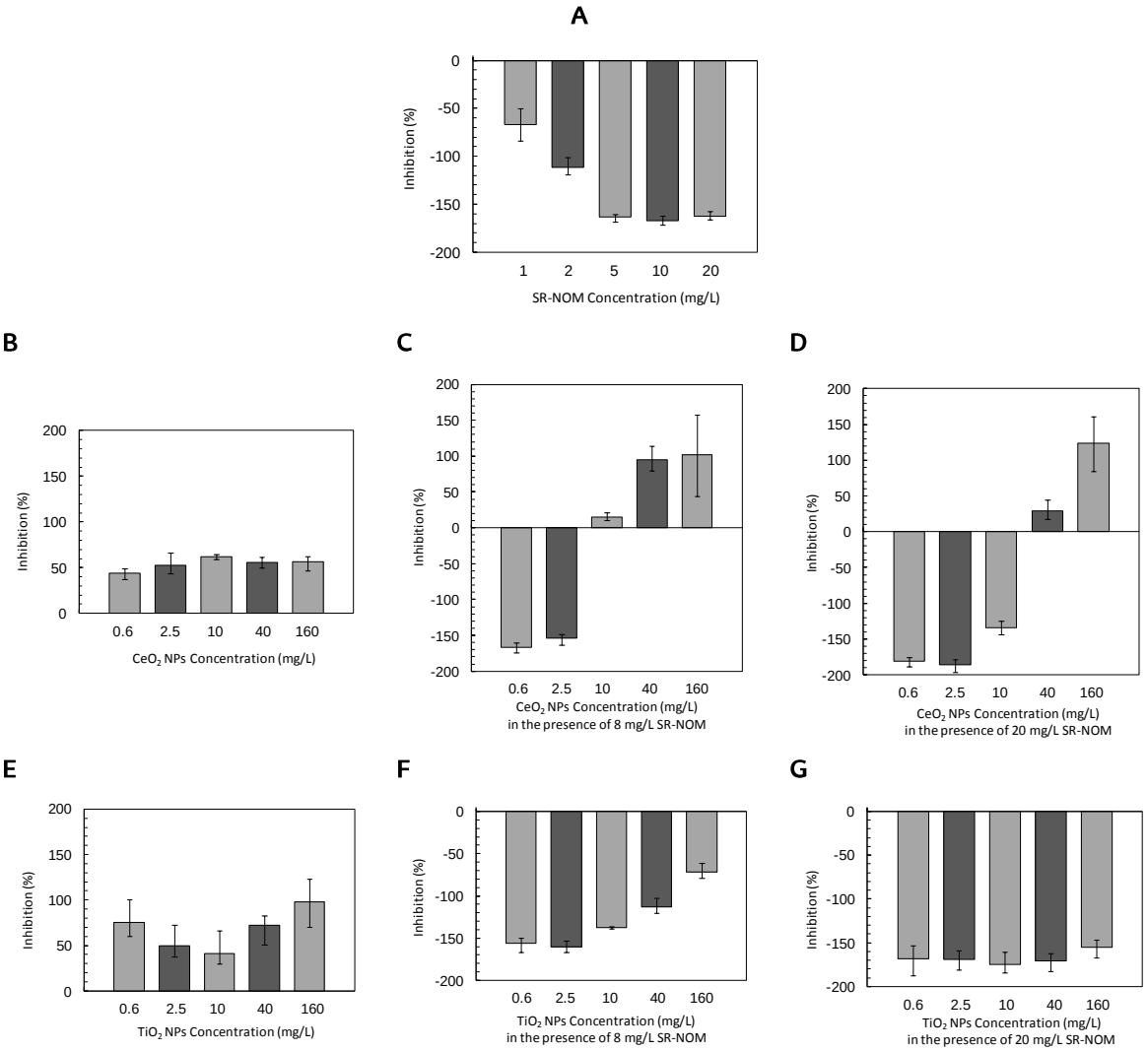

Figure 6.3.21 Histogram comparisons of percent inhibition in average specific growth rates of *Pseudokirchneriella subcapitata* exposed to SR-NOM (A), CeO_2 and TiO_2 NPs in the absence of SR-NOM (B,E), CeO_2 and TiO_2 NPs in the presence of 8 mg/L SR-NOM (C,F) and CeO_2 and TiO_2 NPs in the presence of 20 mg/L SR-NOM (D,G), during 72 h. Error bars represent standard deviation (n=3).

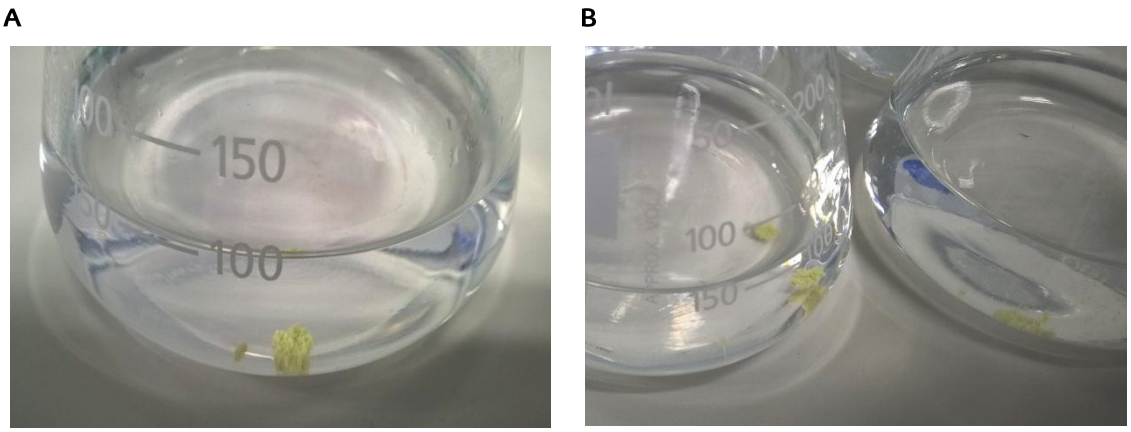

Figure 6.3.22 Clustering of TiO_2 NPs (A) and CeO_2 NPs (B) with algal cells at the end of the ecotoxicity tests in the absence of SR-NOM, for 10 mg/L NPs dispersions.

Increasing SR-NOM concentrations in the algal medium reduced toxicity and resulted in a significant increase of the 50% effective concentration (74.3 mg/L for CeO_2 NPs, no inhibition observed for TiO_2 NPs after adding 20 mg/L SR-NOM). The literature reporting EC50 values for these NPs in the presence of NOM is almost non-existent. However, Van Hoecke et al.[10] obtained 48 h-EC20 values for CeO_2 NPs and *P. subcapitata* between 26.0 and 81.6 mg/L in the presence of organic matter concentrations similar to those used in the present study. Taking into account the different exposure times and endpoints calculated in the current research (72 h-EC50 and EC10), the herein obtained toxicity results were in the same range.

The inhibition histograms corresponding to the tests performed in the absence of organic matter (Figures 6.3.21B and E) showed that representing dose-response curves would have resulted in poor fits with considerable statistical spread. The severe agglomeration of NPs in the algal growth media observed (Z_{ave} ranging from 2 to 3 µm) hindered obtaining reproducible dose-response relationships, as previously reported.[43] In contrast, the enhanced stability provided by SR-NOM led to a better reproducibility of CeO_2 and TiO_2 NPs testing, in the presence of both 8 mg/L and 20 mg/L SR-NOM (Figure 6.3.21C, D, F and G). These histograms showed better fit of dose-response relationships, since inhibitions increased with NP concentrations. Similarly, Cupi et al.[27] proposed that, for some NPs, the presence of NOM may be an important variable to achieve constant exposure conditions, leading to improved reproducibility of their standardized testing.

In the field of nanoecotoxicology it is assumed that stability provided by organic matter results in increased exposure of aquatic biota to NPs.[12] For instance, the

toxicity of TiO_2 NPs to developing zebrafish *Danio rerio* was found to be enhanced after the addition of NOM.[26] Nonetheless, this fact depends on the trophic level of the organism studied. Lin et al.[47] reported that the presence of synthetic HA increased the negative zeta potential of TiO_2 NPs and alleviated their toxicity to the unicellular green algae *Chlorella sp.* Van Hoecke et al.[10] also observed a significant decrease in the toxicity of CeO_2 NPs stabilized with organic matter sampled from a creek towards *P. subcapitata*. Likewise, in the present study the use of organic matter led to a better stability of the dispersed NPs during the tests and at the same time significantly attenuated their adverse effects on algal growth. The colloidal stability provided by SR-NOM to CeO_2 and TiO_2 NPs did not increase their ecotoxicity because bioavailability was influenced by other interaction mechanisms. SR-NOM might 'camouflage' the toxicity of these NPs towards algae due to mechanisms such as complexation, adsorption, electrostatic forces, and oxidation/reduction, as reported by Grillo et al.[12] The reduction in the effects of CeO_2 and TiO_2 NPs suggested that organic matter also acted as stimulating growth factor, taking into account the increase in the growth rates observed in the tests performed independently with SR-NOM (Figure 6.3.21A).

The NOM concentrations tested completely eliminated the toxicity of TiO_2 NPs and caused 'negative inhibitions' on algal growth (Figure 6.3.21F and G). In the case of CeO_2 NPs, this behavior was only observed for concentrations up to 2.5 mg/L and 10 mg/L in the presence of 8 mg/L and 20 mg/L SR-NOM, respectively. The morphology and greater particle surface area of TiO_2 NPs were probably determining factors in their enhanced interactions with SR-NOM (discussed in *Characterization by DLS* subsection), which produced a dramatic reduction in their toxicity compared to that of CeO_2 NPs. Adverse effects ('positive inhibitions') for TiO_2 NPs might be expected by reducing significantly the amount of NOM in dispersions. The correlation represented in Figure 6.3.23 provided some guidance on this concentration, which should be around 2 mg/L SR-NOM or even lower, depending on the TiO_2 NP concentration. However, the representative amounts of organic carbon in freshwater environments for *P. subcapitata* are higher than those corresponding to this concentration of SR-NOM.[27,38] Furthermore, the expected concentrations of MNMs in natural waters of approximately 1 to 100 $\mu g/L^1$ are in the range causing negative inhibitions in the presence of the lowest concentration of SR-NOM tested (up to 2.5 mg/L, Figure 6.3.21C, and up to 160 mg/L, Figure 6.3.21F). Therefore, the 'camouflage' of the ecotoxicity of CeO_2 and TiO_2 NPs towards algae might occur even

for small amounts of NOM and the highest predicted amounts of NPs in aquatic systems. Taking into account the herein obtained results and the stability provided by SR-NOM to metal oxide NPs in algae medium even in the long term,[14] it seems reasonable to introduce SR-NOM into standardized testing methods to assess the ecotoxicity of CeO_2 and TiO_2 NPs towards green microalgae. Further research is needed to analyze its suitability in the ecotoxicological assessment of other nanomaterials, and also to select the specific SR-NOM concentration (or concentration ranges) used in the tests.

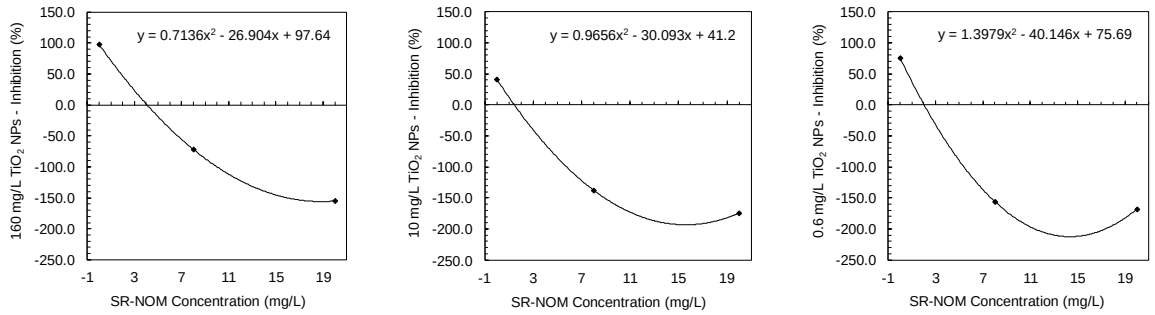

Figure 6.3.23 Correlation curves (fitted to a polynomial form, degree 2) between inhibitions produced by TiO_2 NPs test dispersions and variable concentrations of SR-NOM.

6.3.4. Conclusions

The present study has demonstrated the usefulness of SR-NOM in the assessment of the agglomeration kinetics and ecotoxicity of CeO_2 and TiO_2 NPs towards green microalgae. SR-NOM alleviated their adverse effects on *P. subcapitata* growth, completely in the case of TiO_2 NPs and partially in the case of CeO_2 NPs. Previous studies have evidenced this behavior for other algal species and types of NOM. Furthermore, SR-NOM increased significantly the stability of the NPs in dispersions, which led to a better reproducibility of the toxicity test results. The electrophoretic mobility provided by SR-NOM to CeO_2 and TiO_2 NPs was similar to that previously reported in various river and groundwaters. Therefore, SR-NOM might be a representative sample of what is found in many different ecosystems, thus fulfilling the simulation of realistic environments required for the standardized ecotoxicological assessment of these NPs. The 'camouflage' of the effects of CeO_2 and TiO_2 NPs on algal cells might take place even for small amounts of SR-NOM and the highest predicted amounts of NPs in natural waters.

Chapter 7. Conclusions

Two major contributions have been made by the present study to the toxicological assessment of MNMs in aquatic ecosystems: the optimization of the MNM dispersion methods in the aqueous media of the test organisms and the selection of a reference natural organic matter to conduct the exposures in environmentally realistic conditions.

Multiwalled carbon nanotubes (MWCNTs) were used in the first steps of this research, which led to select the ultrasonic probe as the dispersion method producing an optimization of the energy delivered to the MWCNTs and the lowest agglomeration rates. It was also observed that UV/Vis absorbance measurements used to determine the MWCNT concentrations in aqueous dispersions absolutely depended on the method used to prepare them.

Once the dispersion methods were optimized, the comparison between the agglomeration kinetics and ecotoxicity of MWCNTs in the presence of different organic matters showed an increased stability in *D. magna* medium provided by Suwannee River natural organic matter (SR-NOM). Sigma-Aldrich humic acid

seemed to alter the response of *D. magna* to carbon MWCNTs compared with that shown in the presence of SR-NOM. This fact and the capability of simulating the real conditions in aquatic ecosystems made SR-NOM more appropriate for the ecotoxicological assessment of MWCNTs. In addtition, SR-NOM added to the MWCNT dispersions contributed to observe the important role of their outer diameter and content of impurities in their colloidal stability and ecotoxicity to daphnids.

The advances made in the ecotoxicological assessment of MWCNTs were used to overcome the knowledge gaps on the effects of TiO_2 and CeO_2 nanoparticles on microalgae *P. subcapitata*. Environmentally realistic concentrations of SR-NOM markedly increased the stability of the CeO_2 and TiO_2 NPs in algal medium, which led to a better reproducibility of the toxicity test results. Moreover, the agglomeration kinetics of the CeO_2 and TiO_2 NPs in the presence of SR-NOM were similar to that previously reported in various natural river and groundwaters. Thus, SR-NOM might be a representative sample of what is found in many different ecosystems. Furthermore, SR-NOM alleviated the adverse effects of these NPs on *P. subcapitata* growth, completely in the case of TiO_2 NPs and partially in the case of CeO_2 NPs, suggesting a 'camouflage' of toxicity.

Chapter 8. Future perspectives

Although a comprehensive analysis of the toxicity of every nanomaterial in every environmental compartment poses an expensive and time-consuming task, a previous study is necessary at least for a core set of MNMs at various trophic levels. The present work comprises an ecotoxicological analysis of MWCNTs and TiO_2 and CeO_2 nanoparticles, which constitute a representative part of this core set. This advance may serve as a first step for the development of more efficient testing strategies that should be addressed in the near future, such as computational methods based on Quantitative nanostructure-activity relationship (QNAR) models.

MWCNTs and TiO_2 and CeO_2 nanoparticles represent three of the most relevant MNMs worldwide considering production volumes, market applications and persistence in the environment. Nevertheless, the effects of other MNMs included in the prioritization lists on aquatic organisms (such as SiO_2 and ZnO NPs) should be also studied in the near future.

The addition of a reference natural organic matter to the aqueous media of the organisms has demonstrated to greatly influence the colloidal stability and ecotoxicity of the MNMs studied, and has also allowed conducting the exposures in environmentally realistic conditions. Thus, this substance should be considered to complete the risk assessment of the core set of MNMs.

The stability provided by Suwannee River NOM to the MNM dispersions certainly constitutes a major benefit to increase the reproducibility of the toxicity tests. However, the higher mobility exhibited by CeO_2 and TiO_2 NPs in algal medium in the presence of SR-NOM was accompanied by an attenuation of their adverse effects on algal growth. The modifications of the adverse effects of MNMs observed in the presence of natural organic matter were expected indeed. As mentioned in Chapter 1, NM surfaces can be engineered with a wide variety of designs that enhance their suitability for several industrial applications, but nature will modify their properties when they are released into the environment.

The 'camouflage' of toxicity towards algae observed in the presence of SR-NOM should be thoroughly studied for other MNMs and organisms. Nano-technological products, processes and applications might take advantage of this attenuation of the adverse effects for the development of more environmentally friendly design strategies.

Annexes

ANNEX 1. Instrumental Techniques

1.1. Sonication

The MNMs dispersions were obtained by sonicating with an ultrasonic bath (Sonorex Digitec DT 255/H, BANDELIN) and an ultrasonic homogenizer (VIBRACELL-VCX750, SONICS&MATERIALS) equipped with a standard probe of 136 mm length and 13 mm diameter (Figure 1.1).

Figure 1.1. VIBRACELL-VCX750 sonicator with 13 mm diameter standard probe.[1]

1.1.1. Principles of ultrasonic homogenizer

The ultrasonic generator converts 50/60 Hz voltage to high frequency electrical energy. This alternating voltage is applied to disc-shaped ceramic piezoelectric crystals within the converter, causing them to expand and contract with each change of polarity. The high-frequency longitudinal vibrations are amplified by the probe and transmitted into the liquid as alternating high and low pressure waves.

The pressure fluctuations create millions of micro-bubbles (cavities), which expand during the low pressure phases and implode violently during the high pressure phases. As the bubbles collapse, millions of shock waves, micro jet streams, eddies and extremes in pressures and temperatures are generated and propagated to the surrounding medium. This phenomenon, known as cavitation, lasts a few microseconds and the amount of energy released by each individual bubble is minimal. However, the cumulative amount of energy generated is extremely high,

[1]VIBRACELL-VCX750 Data sheet. SONICS & MATERIALS, Newtown, USA. http://www.sonics.com/lp-vibra.htm Last access: September 2015

causing the disagglomeration of the nanomaterials. Unlike ultrasonic baths, which dissipate the vibrational energy over a large area, the probe focuses the energy to create a concentrated, high intensity sonication zone.

1.1.2. Specifications of the ultrasonic processor

The VIBRACELL-VCX750 processor is equipped with an amplitude control, which continuously evaluates the feedback from the probe, and the frequency and power are automatically adjusted to ensure optimum performance. The specifications of the manufacturer indicate that the vibrations at the probe tip do not decrease as the resistance to the movement of the probe increases, thus critical protocols can be reproduced with confidence.[2]

Probes (also referred to as horns) are one-half wavelength long tools that act as mechanical transformers to increase the amplitude of vibration generated by the converter. They are fabricated from high grade titanium alloy Ti-6Al-4V because of its high tensile strength, good acoustical properties at ultrasonic frequencies, high resistance to corrosion and cavitation erosion, and low toxicity. When driven at its resonant frequency, the probe expands and contracts longitudinally about its center. However, no longitudinal motion occurs at the threaded nodal point, allowing accessories to be connected to the probe at that point. Probes with smaller tip diameters produce greater intensity of cavitation, but the energy released is restricted to a narrower, more concentrated field. Conversely, probes with larger tip diameters produce less intensity, but the energy is released over a greater area. The larger the tip diameter is, the larger the volume that can be processed, but at lower intensity.

Even though ultrasonic vibrations are above the human audible range, ultrasonic processing produces a high pitched noise in the form of harmonics which emanate from the vessel walls and the fluid surface. The sonication processes are performed with the probe inside a sound abating enclosure, which permits reducing the sound by 35 db. The access door permits observation during treatment and protects the operator against accidental splashing.

[2] VIBRACELL High Intensity Liquid Processors-Catalog of products. SONICS & MATERIALS, Newtown, USA, 2015. http://www.sonics.com/lp-vibra.htm Last access: September 2015

1.2. Light Scattering

The stability of the MNMs dispersions was assessed by measuring the variation in scattered light intensity and calculated average zeta-sizes as a function of time. For this purpose, Zeta-average diameter (Z_{ave}), polydispersity index (PDI) values, zeta potentials and electrophoretic mobility (EPM) were obtained by light scattering measurements in a Malvern Zetasizer Nano ZS instrument, considering the data generated from 10 repeated measurements.

1.2.1. Principles of Dynamic Light Scattering (DLS)

Dynamic Light Scattering measures the hydrodynamic diameter of a particle. This is the diameter that determines how fast a particle moves in a suspension through a constant random thermal motion, called Brownian motion. To measure this, DLS uses a laser beam, which is scattered by the nanoparticles when passing through the suspension. The Brownian motion of particles causes fluctuations in the intensity of the scattered light around a mean value. The autocorrelation function of the fluctuations is recorded as a function of time.[3] The principle of dynamic light scattering is that fine particles and molecules that are in constant Brownian motion diffuse at a speed related to their size. Smaller particles diffuse faster than larger particles. The speed of Brownian motion is also determined by the temperature, therefore precision temperature control is essential for accurate size measurement.[4]

DLS, in its standardized form, does not produce particle size distributions, but a light-scattering intensity-weighted average value. The contribution of each particle in the distribution relates to the intensity of light scattered by the particle. A conventional DLS instrument consists of a laser light source, which converges to a focus in the sample using a lens. Light is scattered by the particles at all angles and a single avalanche photodiode detector (APD), traditionally placed at 90º to the laser beam, collects the scattered light intensity. The intensity fluctuations of the scattered light are converted into electrical pulses, which are fed into a digital correlator. This

[3] Linsinger T, Roebben G, Gilliland D, Calzolai L, Rossi F, Gibson N, Klein C. Requirements on measurements for the implementation of the European Commission definition of the term "nanomaterial". JRC Reference Reports. European Commission, Joint Research Centre, Institute for Reference Materials and Measurements. Luxembourg, 2012.

[4] Zetasizer Nano ZS brochure. Malvern Instruments Limited. Worcestershire (UK). http://www.malvern.com/en/products/product-range/zetasizer-range/zetasizer-nano-range/zetasizer-nano-zs/default.aspx Last access: September 2015

generates the autocorrelation function, from which the particle size is calculated (Figure 1.2).[5]

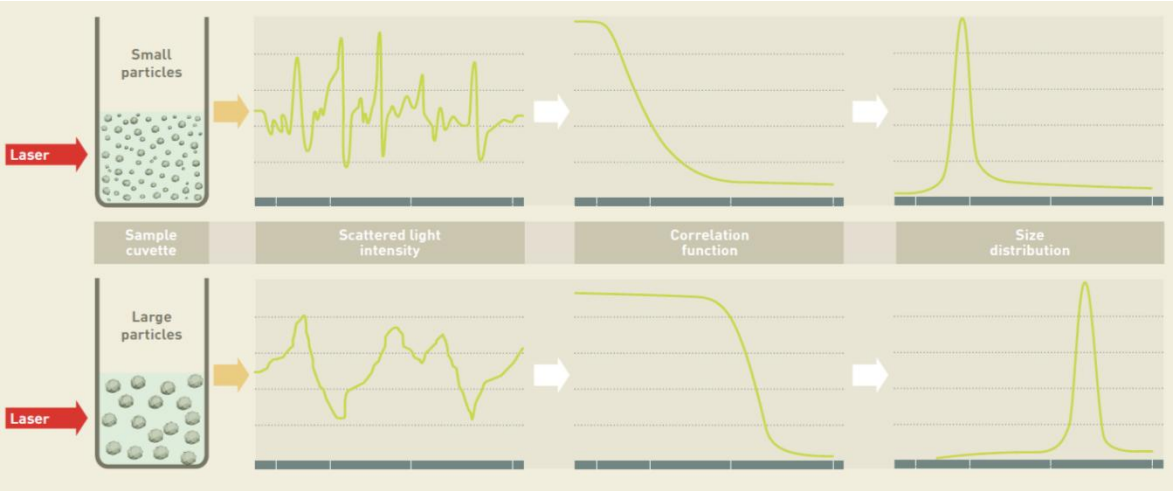

Figure 1.2 The principle of Dynamic Light Scattering. Generation of the correlation function and size distribution.[4]

The Zetasizer Nano ZS uses the Non-Invasive Back-Scatter (NIBS) technology which extends the range of sizes and concentrations of samples that can be measured. The sizing capability in these instruments detects the light scattered at 173°. This is known as backscatter detection. Measuring a larger number of particles eliminates number fluctuations, giving a more stable signal and significantly increasing the largest particle size that can be measured. The optics are not in contact with the sample and hence the detection optics are said to be non-invasive.[5]

DLS is performed in liquids. This limits its use to the analysis of particles which do not dissolve. DLS performs well when dealing with monodisperse samples of suspended nanoparticles with a known refractive index and can measure nanoparticles in the 1 nm to 500 nm range, if these are present in a sufficient concentration. To be able to calculate the hydrodynamic diameter, the temperature and viscosity of the medium are needed.[3]

1.2.2. Principles of Electrophoretic Light Scattering (ELS)

Electrophoretic Light Scattering (ELS) is a technique used to measure the electrophoretic mobility of particles in dispersion or molecules in solution. Its

[5] A basic guide to particle characterization. Malvern Instruments Limited. Worcestershire (UK). http://www.malvern.com/en/support/resource-center/Whitepapers/WP120620BasicGuidePartChar.aspx Last access: September 2015

fundamental physical principle is that of electrophoresis. Dispersions are introduced into a cell containing two electrodes. An electrical field is applied to the electrodes and any charged particles or molecules will migrate towards the oppositely charged electrode. The velocity with which they migrate is known as the electrophoretic mobility and is measured by the laser Doppler technique, and then established theories are applied to calculate the zeta potential (Figure 1.3).[4,5]

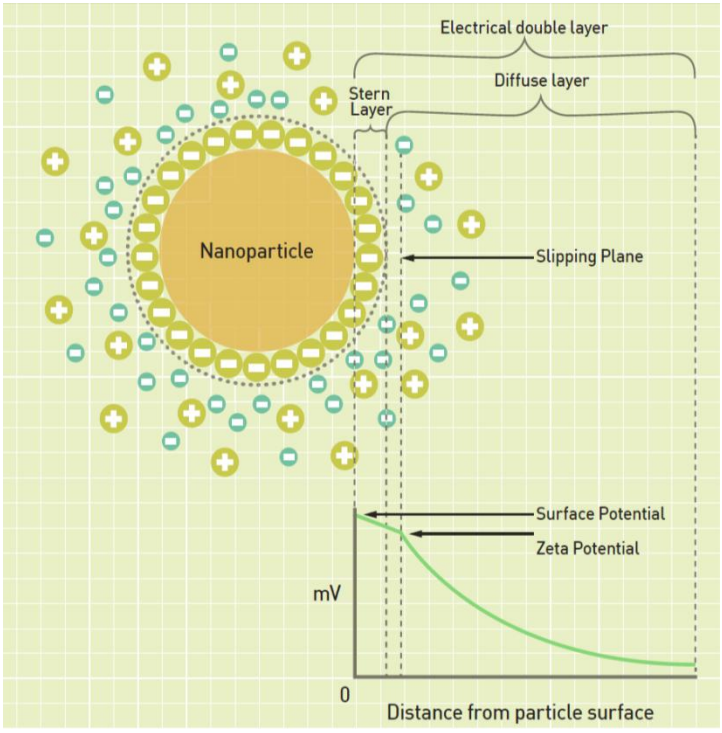

Figure 1.3 Scheme of the charges involved in the zeta potential of a nanoparticle.[4]

The Malvern Zetasizer Nano ZS instrument used combines DLS and ELS to measure both particle size and zeta potential.

1.2.3. Definition of terms

The Z-average size or Z-average mean used in dynamic light scattering is the primary and most stable parameter produced by this technique. The Z-average mean is defined in ISO 13321 and more recently ISO 22412 as the 'harmonic intensity averaged particle diameter'.[6]

The Z-average mean size can be sensitive to even small changes in the sample, e.g. the presence of a small proportion of aggregates. Hence, it will only be comparable

[6] Dynamic Light Scattering. Common terms defined. Malvern Instruments Limited. Worcestershire (UK), 2011. http://www.malvern.com/en/support/resource-center/Whitepapers/WP111214DLSTermsDefined.aspx Last access: September 2015

with the size measured by other techniques if the sample is monomodal (i.e. only one peak), spherical or near-spherical in shape, monodisperse (i.e. very narrow width of distribution), and the sample is prepared in a suitable dispersant. It should be noted that the Z-average is a hydrodynamic parameter and is therefore only applicable to particles in dispersion or molecules in solution.

The polydispersity index is a parameter calculated from a cumulants analysis of the DLS-measured intensity autocorrelation function. In the cumulants analysis, a single particle size mode is assumed and a single exponential fit is applied to the autocorrelation function. The polydispersity describes the width of the assumed Gaussian distribution. The index is dimensionless and scaled such that values smaller than 0.05 are only seen with highly monodisperse standards. Values greater than 0.7 indicate that the sample has a very broad size distribution and is probably not suitable for the dynamic light scattering (DLS) technique. The various size distribution algorithms work with data that falls between these two extremes. The calculations for these parameters are defined in the ISO standard document 13321:1996 E and ISO 22412:2008.

The first order result generated by DLS is an intensity distribution of particle sizes. The intensity distribution is weighted according to the scattering intensity of each particle fraction or family.

The zeta potential is the charge acquired by a particle or molecule in a given medium and arises from the surface charge and the concentration and types of ions in the solution. It is one of the fundamental parameters known to affect dispersion stability. Since particles of similar charge will repel each other, those with high charges will resist flocculation and agglomeration for longer periods making such samples more stable. This means that the stability can be modified by altering the pH, the ionic concentration, the type of ions and by using additives such as surfactants and polyelectrolytes.[4]

1.3. Ultraviolet-visible spectroscopy (UV/Vis)

UV/Vis spectroscopy was selected to analyze the stability and determine the relative concentrations of MNMs. An UV/Vis/NIR spectrophotometer (Lambda 950, PerkinElmer) and quartz cells with 10 mm path length were used for this purpose.

1.3.1. Principles of Ultraviolet-visible spectroscopy

Visible light lies in the wavelength range 4.0-7.0 x 10^{-7} m (Figure 1.4), which is usually quoted in nanometres (400-700 nm). When light is absorbed by a material, valence electrons are promoted from their normal (ground) states to higher energy (excited) states.

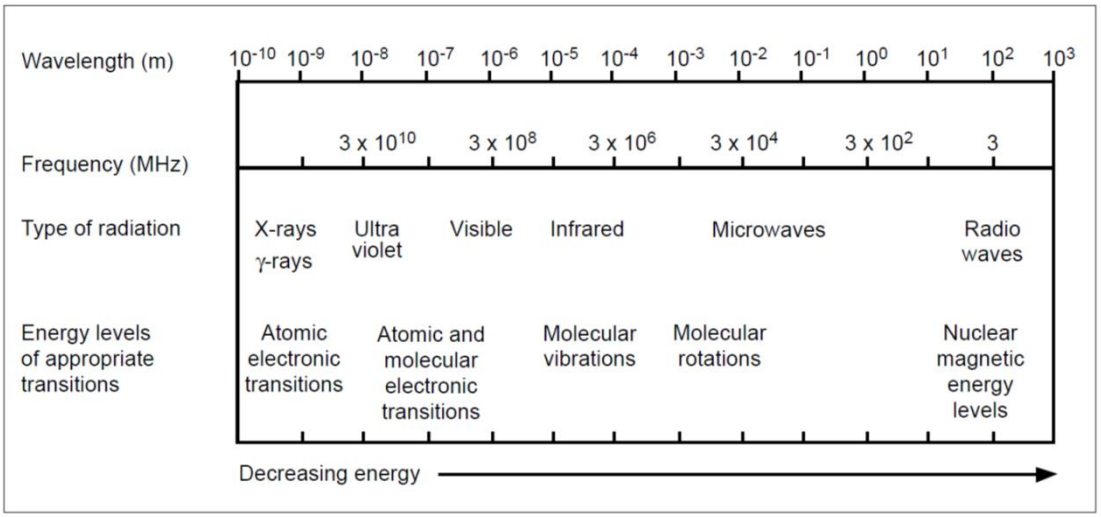

Figure 1.4 Regions of the electromagnetic spectrum.[7]

The energy of visible light depends on its frequency, and is approximately equivalent to 170 kJ mol^{-1} (mole of photons) for red light and 300 kJ mol^{-1} for blue light. The promotion of electrons to different energy levels is not restricted to electromagnetic radiation in the visible part of the spectrum; it can also occur in the ultraviolet region. To encompass the majority of electron transitions the spectrum between 190 and 900 nm is usually considered.

When electromagnetic radiation of the correct frequency is absorbed, a transition occurs from one of the molecular orbitals to an empty orbital, usually an antibonding orbital. The exact energy differences between the orbitals depend on the atoms present and the nature of the bonding system.

To conduct the measurements with the spectrophotometer, it is convenient to have the sample in solution because only small numbers of absorbing molecules are required. The radiation is passed through the sample which is held in a small square-

[7] Royal Society of Chemistry, 2015.
http://www.rsc.org/learn-chemistry/resource/res00001300/ultraviolet-visible-spectroscopy
Last access: September 2015

section cell (1 cm wide internally) in the spectrometer. The Lambda 950 spectrometer features a double-beam, double-monochromator, ratio-recording optical system. The data on the reference is taken first, followed by the sample, and the radiation across the whole range is obtained very quickly. Photocells detect the radiation transmitted and the spectrometer records the absorption by comparing the difference between the intensity of the radiation passing through the sample and the reference cells (Figure 1.5). A computer interprets the two sets of data and plot the spectrum on a chart simultaneously.

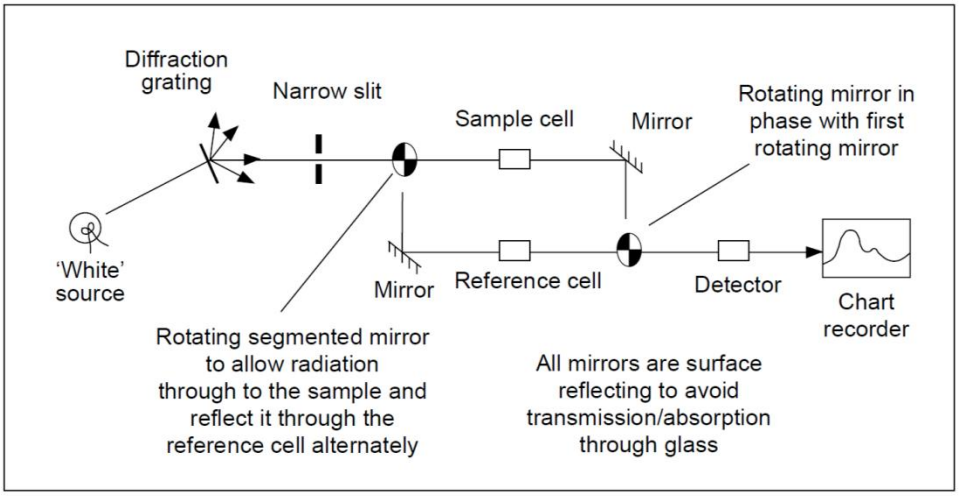

Figure 1.5 Diagram showing the operation of a conventional ultraviolet/visible spectrometer.[7]

1.3.2. Absorption curves

The energies of the orbitals involved in electronic transitions have fixed values, and as energy is quantized, it would be expected that absorption peaks in UV/Vis spectroscopy should be sharp peaks. However this is rarely observed. Instead, broad absorption peaks are seen. This is because a number of vibrational energy levels are available at each electronic energy level, and transitions can occur to and from the different vibrational levels. The situation is further complicated by the fact that different rotational energy levels are also available to absorbing materials.

1.3.3. Specifications and optical system of the UV/Vis/NIR spectrophotometer PerkinElmer Lambda 950

The spectrometer Lambda 950 uses a gridless PMT with Peltier-controlled PbS detector to achieve high-performance testing across the spectral range up to 3300

nm. The UV/Vis resolution reaches 0.05 nm, while the NIR resolution reaches up to 0.20 nm. The components and accessories for sample control include dual sample compartments, a universal reflectance accessory, snap-in integrating spheres and a General-Purpose Optical Bench.[8] The optical components are coated with silica for durability. Holographic gratings are used in each monochromator for the UV/Vis range and the NIR range.[9]

1.4. Scanning Electron Microscopy (SEM)

Electron microscopy was conducted with MNMs dispersions using a ZEISS apparatus (ULTRA PLUS model) and a JEOL apparatus (JSM-7000F model).

1.4.1. Principles of scanning electron microscopy

Light microscopy presents a limit below which it is not possible to resolve an image. In this case, structural information on a specimen can still be obtained by using electrons instead of light. The principles involved are similar, although the operational procedures are different. An electron microscope can be used to obtain magnification in the range 10-10^6 X. Gas molecules scatter electron beams, so the vast majority of studies involving electron microscopy have to be at very low pressures, typically $1.33 \cdot 10^{-3}$-$1.33 \cdot 10^{-5}$ Nm^{-2}. This limits the range of materials that can be studied using this technique to dry, solid specimens that are stable at these very low pressures.

One major similarity between light and electron microscopy is that images can be formed from the electron beam that is transmitted through the specimen or from electron beam that comes back towards the electron beam source, be it a lamp or an electron gun. In the case of electron microscopy, different conditions are necessary for generating and detecting the electron beam. Scanning electron microscopes are useful for displaying images of surface structures, which are generated by secondary electrons.

The most important signals to consider in the electron beam formation and focusing in SEM are the secondary electrons, the backscattered electrons and X-rays.

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[8] LAMBDA UV/Vis and UV/Vis/NIR Spectrophotometers 850/950/1050. Perkin Elmer, Waltham, USA. http://www.perkinelmer.com/catalog/product/id/l950 Last access: September 2015

[9] High-Performance Lambda Spectrometers. Hardware Guide. Perkin Elmer, UK. 2011.

Secondary electrons are usually used to provide the image because the electron beam is not spread out and resolution is often very high, usually in the range 5-20 nm. This type of electron is generated as a result of inelastic scattering of the incident electrons. The secondary electrons have low energies, typically 2-5 eV although they can be as high as 50 eV. The inelastically scattered incident electrons can continue and cause other events.

Backscattered electrons are the primary beam electrons that have been scattered elastically by the nuclei in the sample. These electrons are useful for imaging the atoms in a specimen by atomic number contrast. This is because low atomic number samples give low emissions of backscattered electrons while high atomic number samples give high emissions of these electrons. The backscattered electrons have higher energies than secondary electrons – usually from approximately 50 eV up to the energy of the primary beam electrons.

The electrons can be scattered from relatively deep positions within the sample – typically up to 100 nm, but because the spread of electrons is relatively large, the resolution of any image from these electrons is low (perhaps $2{\cdot}10^{-8}$ m) compared with secondary electron images.

The incident electrons are usually generated by passing an electric current through a filament at the top of a column (other methods exist such as applying a potential to a lanthanum hexaboride single crystal). A voltage, usually in the range 300 V to 40 kV, is applied between the electron source (the cathode) and the rest of the column (the anode). This voltage accelerates the electrons down the column, towards the specimen. Whereas light rays are focused in a light microscope by glass lenses, electrons are focused in an electron microscope by electromagnetic lenses (Figure 1.6).

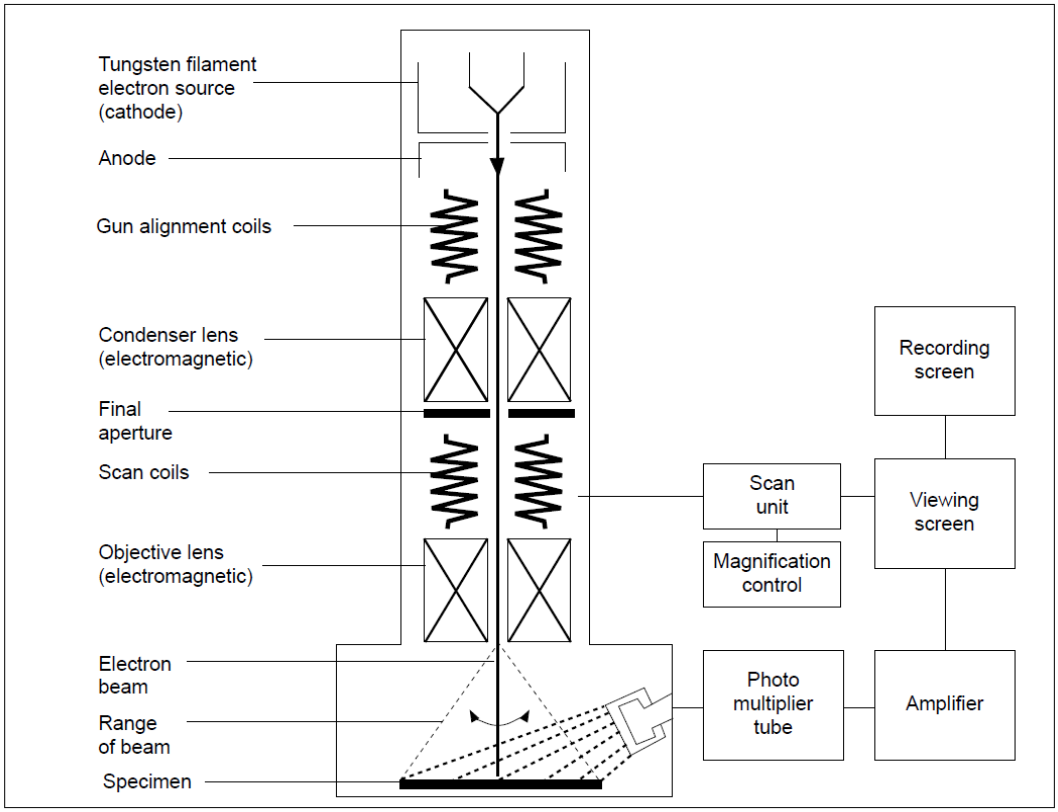

Figure 1.6 The Scanning Electron Microscope (SEM).[10]

The condenser lens is used to collimate the electron beam which the objective lens focuses onto the specimen. Scanning coils are then used to direct the beam across the specimen in a series of parallel lines so that when the parallel scans are put together a two dimensional image is obtained (similar to a domestic television set). Scan rates can be as fast as 25 frames per second for immediate study, or as slow as several minutes per scan if more clearly defined images are required for a photographic record.

The sample is held on a movable stage in a chamber at the base of the column. The stage enables the specimen to be moved in the x, y and z directions, and also allows for tilt and rotational adjustments to be made.

The samples that are non-conductors of electricity have to be treated before they can be studied. Plasma of gold ions can be sputtered onto the sample at very low pressure to form a thin film of gold on its surface. This coating inhibits image distortion by sample charging, and does not normally affect surface detail because

[10] Royal Society of Chemistry, 2015.
http://www.rsc.org/learn-chemistry/resource/res00001302/electron-microscopy
Last access: September 2015

the gold coating can only be detected at relatively high magnifications. Gold is often used because it is an excellent electrical conductor and being a heavy metal, has a high secondary and back scattering electron yield.

1.4.2. Image formation

Secondary electrons are useful for high resolution imaging. They are attracted by a grid, typically set at +200 to +600 V potential, in front of a scintillation detector. They are further accelerated by a potential of about 10 kV onto the scintillation detector surface, where their energy is converted to visible light. The light emitted passes down a perspex light guide to a photomultiplier tube where it is converted to an electrical current. This signal can be amplified to produce an image on a cathode ray tube (a television screen). A large number of secondary electrons results in a bright image on the screen.

Photographic images are produced by placing a camera in front of a suitable screen and moving images can also be recorded by using videotape. Images can be clarified by removing unwanted background 'noise' with the aid of a computer.

The magnification can be changed by changing the area of the sample scanned while keeping the screen size constant. A large magnification is achieved by scanning a very small area of the sample. The images obtained have an advantage over light microscopy images because they have a 'three dimensional' quality and have an appreciably greater depth of field.

1.5. Fluorescence intensity

Fluorometric determinations were conducted to obtain the chlorophyll-a concentrations. Extracted chlorophyll permitted the estimation of the biomass of the algal cultures in the presence of MNMs, which interfere with measurements of culture density normally made by optical absorbance.

The fluorescence of the samples was determined in arbitrary units on a microplate reader (FLUOstar OPTIMA, BMG-LABTECH,) with an excitation wavelength of 430 nm and a measured emission wavelength of 670 nm. The needed sub-sample volume was 350 µL in 96-well polypropylene black microplates (Greiner Bio One).

1.5.1. Principles of fluorescence

At room temperature most molecules occupy the lowest vibrational level of the ground electronic state, and on absorption of light they are elevated to produce excited states. Figure 1.7 below shows absorption by molecules to produce either the first, S_1, or second S_2, excited state.

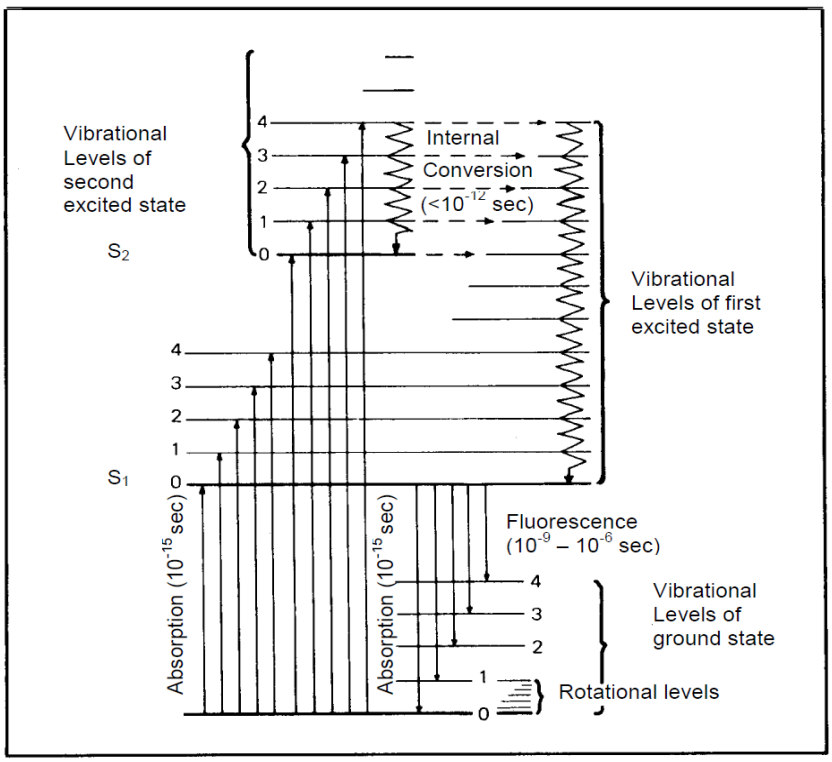

Figure 1.7 Transitions giving rise to absorption and fluorescence emission spectra.[11]

Excitation can result in the molecule reaching any of the vibrational sub-levels associated with each electronic state. Since the energy is absorbed as discrete quanta, this should result in a series of distinct absorption bands. However, the simple diagram above neglects the rotational levels associated with each vibrational level and which normally increase the number of possible absorption bands to such an extent that it becomes impossible to resolve individual transitions. Therefore, most compounds have broad absorption spectra except for those where rotational levels are restricted (for example, planar, aromatic compounds).

Having absorbed energy and reached one of the higher vibrational levels of an excited state, the molecule rapidly loses its excess of vibrational energy by collision

[11] An Introduction to Fluorescence Spectroscopy. PerkinElmer, UK. 2000.

and falls to the lowest vibrational level of the excited state. In addition, almost all molecules occupying an electronic state higher than the second undergo internal conversion and pass from the lowest vibrational level of the upper state to a higher vibrational level of a lower excited state which has the same energy. From there the molecules again lose energy until the lowest vibrational level of the first excited state is reached.

From this level, the molecule can return to any of the vibrational levels of the ground state, emitting its energy in the form of fluorescence. If this process takes place for all the molecules that absorbed light, then the quantum efficiency of the solution will be a maximum (unity). However, if any other route is followed, the quantum efficiency will be less than 1 and may even be almost zero.

The 0-0 transition, from the lowest vibrational level in the ground electronic state to the lowest vibrational level in the first excited state, is common to both the absorption and emission phenomena, whereas all other absorption transitions require more energy than any transition in the fluorescence emission. We can therefore expect the emission spectrum to overlap the absorption spectrum at the wavelength corresponding to the 0-0 transition and the rest of the emission spectrum to be of lower energy, or longer wavelength (Figure 1.8).

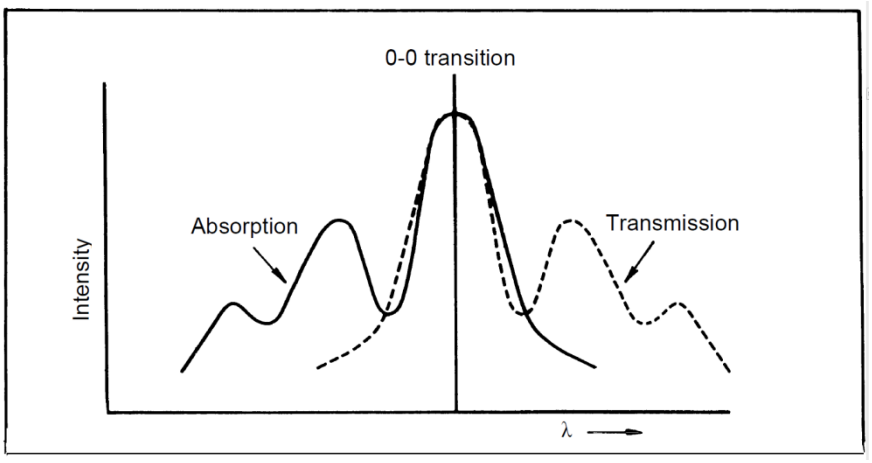

Figure 1.8 Idealized absorption and emission spectra.[11]

In practice, the 0-0 transitions in the absorption and emission spectra rarely coincide exactly. The difference represents a small loss of energy by interaction of the absorbing molecule with surrounding solvent molecules.

The absorption of energy to produce the first excited state does not perturb the shape of the molecule greatly and this means that the distribution of vibrational levels is very similar in both the ground and first excited states. The energy differences between the bands in the emission spectrum will be similar to those in the absorption spectrum and frequently the emission spectrum will be approximate to a mirror image of the absorption spectrum. Since the emission of fluorescence always takes place from the lowest vibrational level of the first excited state, the shape of the emission spectrum is always the same, despite changing the wavelength of exciting light.

A plot of emission against wavelength for any given excitation wavelength is known as the emission spectrum. If the wavelength of the exciting light is changed and the emission from the sample plotted against the wavelength of exciting light, the result is known as the excitation spectrum. Furthermore, if the intensity of exciting light is kept constant as its wavelength is changed, the plot of emission against exciting wavelength is known as the corrected excitation spectrum.

The quantum efficiency of most complex molecules is independent of the wavelength of exciting light and the emission will be directly related to the molecular extinction coefficient of the compound; in other words, the corrected excitation spectrum of a substance will be the same as its absorption spectrum.

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ANNEX 3. Publications and dissemination activities

Cerrillo C, Barandika G, Igartua A, Areitioaurtena O, Marcaide A, Mendoza G. **2015**. Ecotoxicity of multiwalled carbon nanotubes: Standardization of the dispersion methods and concentration measurements. *Environ Toxicol Chem* 34:1854-1862. Q1 (45/223; ENVIRONMENTAL SCIENCES).

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Publications

Publication 1

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ECOTOXICITY OF MULTIWALLED CARBON NANOTUBES: STANDARDIZATION OF THE DISPERSION METHODS AND CONCENTRATION MEASUREMENTS

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Abstract: There are currently a variety of applications for multiwalled carbon nanotubes (MWCNTs), but considerable concerns exist regarding their release into the environment. Their potential accumulation by aquatic organisms could lead to transfer throughout food chains. Considering the divergences in experimental data published on the ecotoxicity of carbon nanotubes, further research is required. The dispersion of MWCNTs in aqueous culturing media of organisms as well as the determination of concentrations are relevant aspects to obtain accurate ecotoxicity results. Ultraviolet-visible spectroscopy is one of the most reported techniques to analyze concentration quickly and economically, but the methodologies to prepare dispersions and selecting the wavelengths for ultraviolet-visible measurements have not yet been clearly defined. The present study demonstrates that dispersion procedures influence absorbance, and an approach to determine the most appropriate measurement wavelength is proposed. Ecotoxicity tests with MWCNTs were performed on *Vibrio fischeri* bacteria, and divergences in the results were observed with respect to those previously reported. The present study contributes to the attempt to overcome the lack of standardization in the environmental assessment of MWCNTs. *Environ Toxicol Chem* 2015;34:1854–1862. © 2015 SETAC

Keywords: Ecotoxicity Multiwalled carbon nanotube Humic acid Sonication Ultraviolet-visible spectroscopy

INTRODUCTION

Nanotechnology is playing a key role in the development of goods and services around the world and fostering the competitiveness of industries in the knowledge economy. Within nanomaterials, the unique physical, chemical, electrical, and mechanical properties of carbon nanotubes (CNTs) are promoting the increase in the number of applications in different fields (e.g., chemistry, electronics, energy, materials science, medicine) [1,2]. Large-scale production and applications of CNTs are steadily increasing. Thus, there are considerable concerns over their inevitable release into the environment and human exposure to them because their accumulation by aquatic organisms could lead to transfer throughout food chains [3–5]. In addition, the limited understanding of the environmental, health, and safety aspects of CNTs poses a threat to their potential applications, considering that experimental data related to their toxicity at different levels have been published [6,7] and that the results are often divergent. This inconsistency could be a consequence of factors such as impurities, surface modifications, structure, and exposure routes [4]. Therefore, more attention to toxicology research on them is required to achieve a systematic understanding of their real toxicity.

The number of industrial-scale facilities for the relatively low-cost production of multiwalled CNTs (MWCNTs) is growing steadily [8,9], and their release into the environment is foreseen to be greater than that of single-walled CNTs (SWCNTs). Thus, research on MWCNT toxicity is considered to be more imperative.

A relevant issue in ecotoxicity studies is the solubility of toxicants in aqueous culturing media. An important obstacle must be faced regarding this issue because CNTs exhibit a hydrophobic nature and a tendency to form agglomerates, which hinders the preparation of stable dispersions in water [10]. Many effective methods, both physical and chemical, have been proposed to disperse CNTs in aqueous solutions, such as stirring, sonication, and addition of surfactants [11]. Nevertheless, the use of these treatments affects the inherent properties of CNTs [12] and, therefore, the interactions they might have with living organisms [13–15]. Because of this, minimizing the effect of these physicochemical treatments on the CNT characteristics becomes necessary. With regard to physical methods, sonication time, frequency, and power have been proven to modify the attributes of nanotubes, such as length and, hence, toxicity [16,17]. Therefore, the reduced energy delivered by sonication baths can be thought to be more appropriate than that of sonication probes. In relation to chemical treatments, selection of biocompatible dispersants is required to avoid the alteration of the toxicity effect of nanotubes. Several surfactants did not show toxicity in living organisms at low concentration levels [18]. However, the most suitable dispersants for ecotoxicity studies are those present naturally in environmental media, such as natural organic matter (NOM) and its major component, humic acid [19,20], to reproduce realistic environmental conditions in assays.

Once the dispersion procedure has been selected, determining CNT concentrations in dispersions is a critical issue to obtain accurate toxicity values. Different techniques are currently available to estimate the dispersion state and even stability of CNTs (conventional microscopy including optical microscopy, atomic force microscopy, scanning electron microscopy, and

All Supplemental Data may be found in the online version of this article.

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transmission electron microscopy; dynamic light scattering; and zeta-potential measurements) [10]. However, those methods are, in most cases, qualitative, and the effect of dispersion cannot be evaluated precisely. In addition, photoluminescence and ultraviolet-visible (UV-visible) spectroscopies have been used to determine quantitatively CNT concentrations. In fact, UV-visible is one of the most reported techniques in the last 10 yr, given its rapidity, its low cost, and the possibility of concentration measurements of both SWCNT and MWCNT dispersions [18,21,22]. However, UV-visible absorbance poses some challenges that have not been solved clearly to date. A key factor in the determination of concentrations by UV-visible spectroscopy is the preparation of calibration curves, based on dispersions with previously known concentrations [20]. Some studies on this issue do not use exactly the same methods (variable sonication processes) to prepare samples for calibration curves and samples for toxicity assessment [5,23,24]. This fact could lead to misleading results in concentration values because different parameters or preparation techniques result in different dispersion states [11,25]. Furthermore, variations in the wavelengths selected for absorbance measurements are observed. Previous studies have shown that absorbance peaks, achieved at established wavelengths, are linearly correlated with the MWCNT concentration [20,24]. Measurement wavelengths reported are 800 nm [18,20,23,26], 600 nm [27,28], 530 nm [29], 500 nm [21,30], 298 nm [25], and 260 nm [11].

The present study focused on making progress in the field of MWCNT ecotoxicology by improving the accuracy of toxicity assessments. The first objective was to analyze the adequacy of UV-visible spectroscopy for the preparation of calibration curves to determine the concentration of MWCNTs in dispersions. Because some studies on this issue use different sonication processes to prepare samples for calibration curves and toxicity assessment, the present investigates whether those different techniques produce the same UV-visible absorbance results. Furthermore, considering that variations in the wavelengths selected for absorbance measurements are observed in previously mentioned works, we propose a procedure to select an appropriate wavelength for each type of MWCNT. After optimization of the dispersion parameters, ecotoxicity tests for MWCNTs were performed on *Vibrio fischeri* bacteria. This aquatic organism was selected taking into account that bacteria constitute the lowest organism level and the entrance to the food web in many ecosystems. The ecotoxicity data obtained should be considered in terms of reliability because the selection of the most appropriate dispersion methods permits standardization of the study of the environmental effects of MWCNTs.

MATERIALS AND METHODS

Materials for dispersions

Two different commercial MWCNTs, CNT-N and CNT-A, were used as received from the manufacturers. Both were produced via catalytic chemical vapor deposition, and their physical descriptions and commercial sources are detailed in Supplemental Data, Table S1. Some relevant differences were observed in the physical properties of the nanotubes studied (outer diameter, length, and percentage of impurities). Scanning electron microscopy was performed directly on dry CNT powder, using a Zeiss apparatus (Ultra Plus model) with a magnification of 48 000× (see Figure 1).

Humic acid was purchased from Sigma-Aldrich Química and used without any further purification. Ultrapure water (MilliQ) was produced using a water filtration system from Millipore Iberica to prepare all the dispersions.

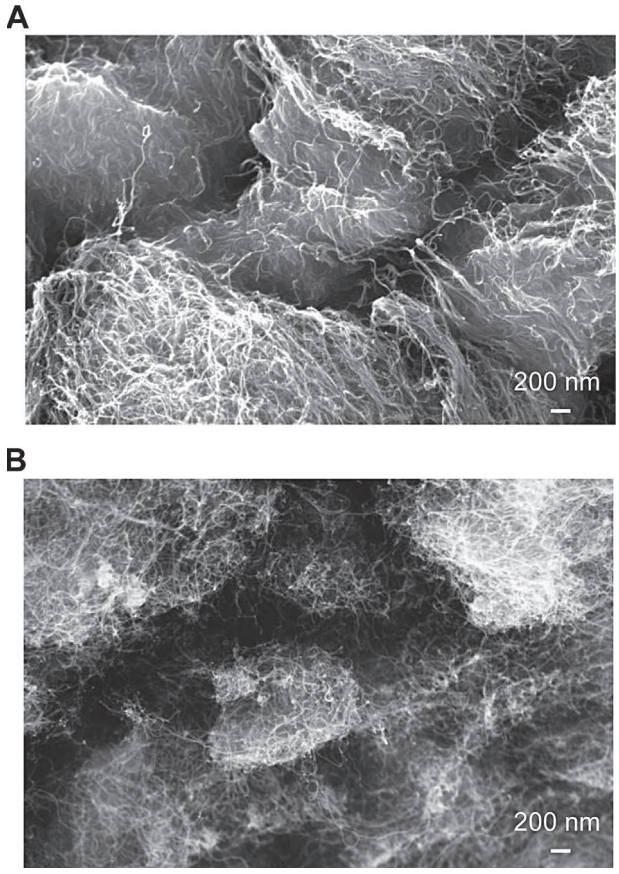

Figure 1. Scanning electron microscopic images of multiwalled carbon nanotubes: (**A**) CNT-N and (**B**) CNT-A. Magnification 48 000×. CNT = carbon nanotube.

Dispersion preparation and characterization

Dispersions for calibration curves. Humic acid was selected as the model NOM to prepare the dispersions. The concentrations of humic acid must produce the dispersion of the required amount of MWCNTs. At the same time, these concentrations must be nontoxic to avoid alteration of the ecotoxicity of nanotubes. To ensure the appropriate experimental concentrations of MWCNTs, dispersion tests were performed. The results showed that 30 mg/L of CNTs could be dispersed in 100-mg/L humic acid solutions. Concentrations of 100 mg/L humic acid were experimentally observed to cause no inhibition on *V. fischeri* bacteria, and therefore they were selected to prepare dispersions.

Humic acid solutions were prepared by adding 100 mg/L humic acid into ultrapure water. They were mixed constantly for 48 h at 20 ± 2 °C by means of magnetic stirring, as previously reported [7,26]. This time was sufficient to achieve complete dissolution of humic acid. Thus, further centrifugation or filtration steps to obtain the supernatants were not necessary.

The sonication process for calibration dispersions was carried out as previously described using an ultrasonic homogenizer [31] (Vibracell-VCX750; Sonics & Materials) with a standard probe (136 mm length, 13 mm diameter), at an operating frequency of 20 kHz, pulsing operating mode of 1 s on/1 s off, and output power fixed at 750 W at 60% amplitude. Dispersions were prepared by mixing the corresponding amount

of MWCNTs with 25-mL of 100-mg/L humic acid solution in 200-mL glass beakers and sonicating for 2.5 min. Sonication was repeated 3 times more, adding 25 mL of humic acid solution at each stage, until the volume was adjusted to achieve a CNT concentration of 30 mg/L. The beaker was held in an ice bath during sonication to prevent a rise in the temperature of the sample and covered with Parafilm (plastic paraffin film) to avoid evaporation.

Calibration standards were made by diluting the 30-mg/L dispersions with 100-mg/L humic acid solution, obtaining 12 more levels: 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, 2.5 mg/L, 2 mg/L, 1.5 mg/L, 1 mg/L, 0.5 mg/L, 0.25 mg/L, and 0.1 mg/L.

Dispersions for verification of calibration curves. The dispersions for verification of calibration curves were also prepared with 100-mg/L humic acid solutions. Three different concentrations in the same range as the calibration dispersions were selected to prepare: 2.5 mg/L, 5 mg/L, and 10 mg/L. These concentrations were high enough to avoid accuracy errors in weighing CNTs but not too high to obtain stable dispersions, according to previously described methods [23,25].

The sonication process was carried out with an ultrasonic bath (Sonorex Digitec DT 255/H) at an operating frequency of 35 kHz and 160 W output power, filling the bath with cool water (15 °C) at the same level as that inside the sample bottles. Dispersions were prepared by mixing the corresponding amount of MWCNTs with 50 mL of 100-mg/L humic acid solution in 250-mL glass flasks and sonicating for 15 min. Sonication was repeated 3 times more, adding 50 mL of humic acid solution at each stage, until the volume was adjusted to achieve the required CNT concentrations for verification. Three flasks were sonicated at the same time in each experiment to ensure the same level of energy received by the dispersions.

Calculation of the total amount of energy delivered by the sonication methods from calorimetry. Given the importance of the dispersion techniques in the present study, the delivered acoustic energy supplied by the ultrasonicators was calculated using the calorimetric method described by Taurozzi et al. [32]. A study on this subject [11] has demonstrated that there is a minimum energy required to disperse the optimum amount of MWCNTs in aqueous solution and that their dispersion behavior is also determined by parameters such as the concentration of CNTs and the ratio of CNTs to dispersant. Taking into account the results obtained in that work and the concentrations used in the present study, we established that the total amount of energy delivered to the dispersions prepared should not be higher than 30 kJ to prevent damaging and cutting effects on CNTs. Considering the sonication times selected, the total amount of energy delivered (E) was 12.68 kJ for the sonicator probe and 26.20 kJ for the sonicator bath. Because a similar energy for both sonication methods was required to obtain comparable results, the sonication time for the sonicator probe was duplicated for the preparation of dispersions (from 5 min to 10 min), and the total amount of energy delivered was finally 25.35 kJ. These values were in accordance with the maximum specified above to prevent harmful effects on CNTs. Additional details on this calculation are provided in the Supplemental Data.

Dispersion characterization. Two factors affect the UV-visible absorbance ability of MWCNTs: their intrinsic properties and the agglomeration rate. If the size of the agglomerates is comparable to the wavelength of the light, the intrinsic properties are the main influencing factor. If the size of the MWCNT agglomerates is much larger than the wavelength, the agglomeration rate is the main influencing factor [21]. Therefore, absorbance peaks vary depending on both the features of the CNTs and the methods employed to prepare dispersions. Thus, a spectral analysis is always necessary to check the absorbance maxima of the studied nanotubes.

Absorbance spectra for 30-mg/L dispersions were obtained immediately after sonication, and a spectral analysis of the humic acid solution was performed to check that it did not alter the baseline of MWCNT absorbance spectra [20]. Absorbance spectra were obtained using a UV-visible spectrophotometer (Lambda 950; PerkinElmer) and quartz cells with a 0.2-mm path length. Although the initial operating range of the spectrophotometer was 200 nm to 2000 nm, because no remarkable changes in the spectra were appreciated over 1200 nm, the final wavelength range selected in the present study was 200 nm to 1200 nm, considering also the data reported on this issue [11]. The wavelength for calibration curve measurements was selected considering the absorbance spectra of MWCNTs and humic acid (see *Results and Discussion*). Measurements for calibration curves were conducted using a Jenway 6300 spectrophotometer, which provided more speed to obtain absorbance at a specific wavelength. This equipment operates at 320 nm to 1000 nm wavelength with 10-mm path length quartz cells.

Prior to the UV-visible absorbance measurements, the dispersions were characterized with the aim of analyzing their stability by size distributions and rate of agglomeration. Thus, these properties could be compared for both types of dispersions, and it could be checked whether the sonication parameters selected (time and amplitude) were appropriate. The Z-average diameter (Z_{ave}) and polydispersity index were obtained by dynamic light scattering measurements in a Malvern Zetasizer Nano ZS instrument, considering the data generated from 10 repeated measurements.

Selection of the dispersion method and ecotoxicity tests

Based on the results of the dispersion characterization, the most appropriate sonication method to prepare dispersions for ecotoxicity tests was determined. To check the effective deagglomeration efficiency of MWCNTs in these dispersions, dynamic light scattering measurements were carried out immediately after their preparation. Moreover, the concentrations of CNTs were measured by UV-visible absorbance to check whether these values corresponded to those of calibration curves.

Vibrio fischeri bacteria were selected to carry out the ecotoxicity tests, considering that very few studies have reported data of CNT toxicity on this microorganism [33,34]. The assays were performed on LUMIStox 300 photometer controlled by LUMISsoft IV software (Dr. Lange), according to UNE-EN ISO 11348-2:2009 [35]. *Vibrio fischeri* produces light as a by-product of its cellular respiration, and the assay results were the toxicant effective concentrations causing 20% (EC20) and 50% (EC50) inhibition in light emission. The exposure time between dilution rows of the samples and bacteria was 30 min, and the reference substance used was $K_2Cr_2O_7$ (Sigma-Aldrich Química). Three independent tests were performed, and standard deviation values for EC20 and EC50 were calculated.

RESULTS AND DISCUSSION

Selection of the measurement wavelength

As mentioned in *Materials and Methods*, the spectral analysis was intended to determine MWCNT absorbance peaks and whether there was any alteration in their spectra as a result of the

presence of humic acid in dispersions, with the aim of selecting the measurement wavelengths for each CNT. For this purpose, the spectra of 30 mg/L MWCNT dispersions were obtained in 2 different ways: 1) considering the 100-mg/L humic acid solution effect, taking it as a background substance, and subtracting its absorbance by the "autozero" function of the spectrophotometer and 2) measuring absorbance of dispersions by taking ultrapure water as a background solution. Thus, we could analyze whether the absorbance of both humic acid and MWCNTs was additive, as previously reported [5], and whether this fact was noticed along the whole spectrum. Figure 2 shows absorbance peaks of humic acid and MWCNTs in arbitrary units (a.u.).

Absorbance peaks of humic acid and MWCNTs were observed at similar wavelengths. The humic acid absorbance maximum was achieved at 206 nm (Figure 2, continuous line), and those for CNT-N and CNT-A (dotted lines) were at 240 nm and 241 nm, respectively. Considering the spectra of dispersions with humic acid (dashed lines), a shift to the left was observed at absorbance peaks, decreasing to 227 nm and 222 nm. Although these maxima were higher than those for CNT-N and CNT-A without humic acid, humic acid involved alteration of the UV-visible absorbance of MWCNT dispersions. The absorbances of CNTs and humic acid were not fully additive because a deviation of the calculated values was obtained with respect to theoretical ones at the CNT peak maxima (see Figure 2). Because of this, the wavelengths for calibration curves were moved to other spectral values, different from the absorbance peaks. Dispersions with humic acid and those in which its effect was subtracted overlapped their absorbance spectra in a specific wavelength (Figure 3). This fact could imply that, at this wavelength, the humic acid effect was nonexistent. Those wavelengths were 535 nm for CNT-N and 537 nm for CNT-A, similar to the previously reported wavelength of 530 nm [29]. Hence, they were selected to perform calibration curve measurements and the corresponding verifications.

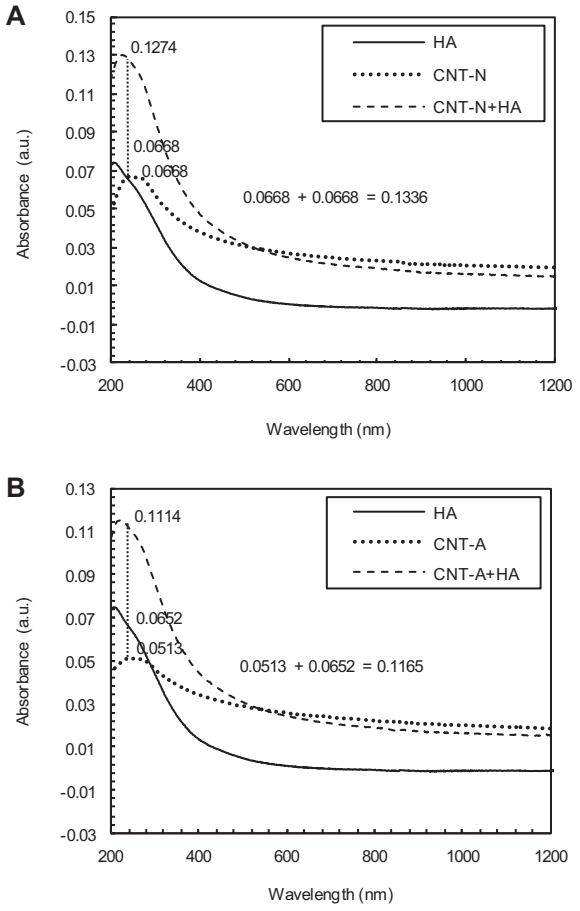

Figure 2. Ultraviolet-visible spectra of humic acid solution (continuous lines), multiwalled carbon nanotube dispersions considering humic acid as a background solution (dotted lines), and multiwalled carbon nanotube dispersions considering ultrapure water as a background solution (dashed lines) for CNT-N (**A**) and CNT-A (**B**). CNT = carbon nanotube; HA = humic acid.

Calibration curves and verification

Taking into account the wavelengths selected to carry out measurements, absorbance values were obtained for each dilution level and type of CNT studied (Supplemental Data, Table S2). Dispersions were measured taking ultrapure water as a background substance.

Previous studies have reported calibration curves with absorbances that range from 0.1 a.u. to 1.1 a.u. [19] and from 0.1 a.u. to 0.5 a.u. [21]. The obtained absorbance values (Figure 4) were in the same range as those previously reported. Variations were caused by the different ultrasonic treatments used, the types and concentrations of CNTs and dispersants, and the spectrophotometers employed to perform absorbance measurements. Furthermore, considering that the absorbance range of the spectrophotometer was between –0.300 and 1.999, it was not possible to obtain absorbance values for the highest concentrations (30 mg/L for CNT-N and 20 mg/L, 25 mg/L, and 30 mg/L for CNT-A; Supplemental Data, Table S2; Figure 4).

As explained in *Materials and Methods*, absorbance values for calibration were only verified at specific concentrations: 2.5

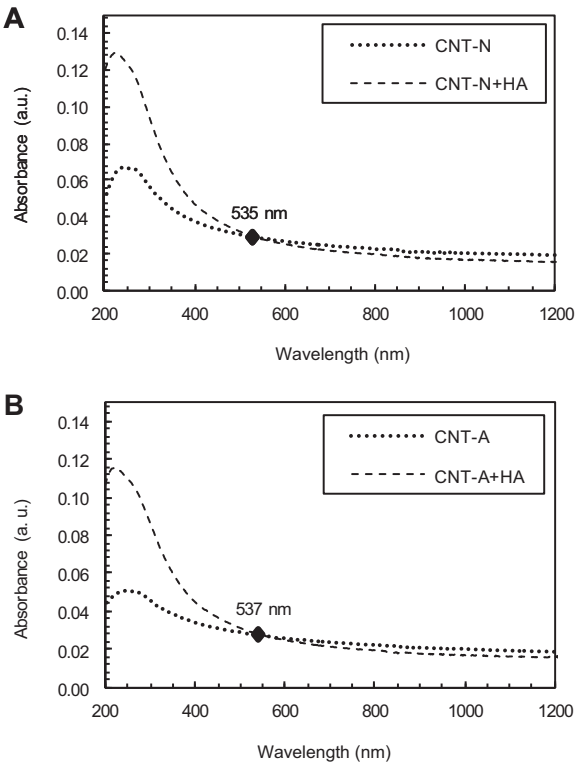

Figure 3. Ultraviolet-visible spectra of multiwalled carbon nanotube dispersions considering humic acid as a background solution (dotted lines) and multiwalled carbon nanotube dispersions considering ultrapure water as a background solution (dashed lines) for CNT-N (**A**) and CNT-A (**B**), and the measurement wavelengths selected to perform calibration curves and verifications. CNT = carbon nanotube; HA = humic acid; a.u. = arbitrary unit.

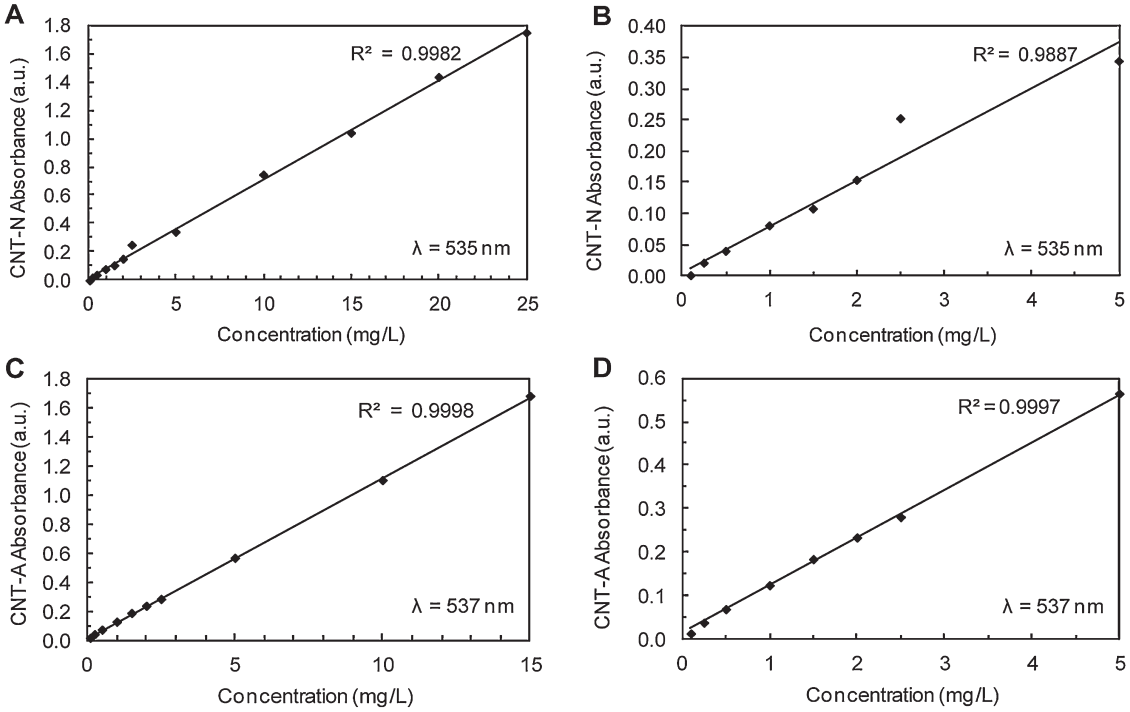

Figure 4. Calibration curves obtained from absorbance of carbon nanotube dispersions in different ranges of dilution levels, from 0.1 mg/L to 30 mg/L (**A,C**) and from 0.1 mg/L to 10 mg/L (**B,D**). Straight lines are linear least-squares fit to the data. CNT = carbon nanotube; a.u. = arbitrary unit.

mg/L, 5 mg/L, and 10 mg/L. Table 1 includes absorbance values obtained for these dispersions at the same wavelengths used to prepare calibration curves and taking ultrapure water as the background substance.

For both CNT-N and CNT-A, the verification values showed considerable differences with respect to calibration values. Verification dispersions did not achieve the absorbance obtained for calibration dispersions, and differences were higher as concentrations increased over 5 mg/L. As mentioned in *Materials and Methods*, if the size of the MWCNT agglomerates is much larger than the wavelength used for absorbance measurements, the former is the main influencing factor affecting the UV-visible absorbance ability of the MWCNTs [21]. The present study's results revealed that different ultrasonic treatments, considering the same concentration of CNTs, could not result in the same absorbance results. This could be attributed to the fact that the size of the agglomerates obtained after ultrasonic treatment was larger than the wavelength used in UV-visible measurements, and for dispersions prepared by sonication bath, CNTs remained more agglomerated than for dispersions prepared by ultrasonic probe. To corroborate this hypothesis, dynamic light scattering analysis was carried out.

Dispersion characterization by dynamic light scattering

Dynamic light scattering characterization provided relevant data to analyze and compare the stability of dispersions by the size distributions and rate of agglomeration. The concentrations analyzed corresponded to 30 mg/L, 10 mg/L, 5 mg/L, and 2.5 mg/L for calibration curve dispersions and to 10 mg/L, 5 mg/L, and 2.5 mg/L for verification dispersions. Table 2 shows the Z_{ave} sizes and polydispersity indexes obtained, and the size distribution graphs of 10 mg/L dispersions are included in Figure 5. Supplemental Data, Figures S2 to S5, provide the rest of the size distribution graphs obtained.

As can be seen in Table 2 and Figure 5, substantial differences for Z_{ave} diameters and polydispersity index existed between calibration and verification dispersions. In the case of the latter, those parameters were quite higher for both types of MWCNTs. Thus, for dispersions prepared by sonication bath, CNTs remained more agglomerated than for dispersions prepared by ultrasonic probe, and the hypothesis established in the previous subsection (*Calibration curves and verification*) was confirmed. The relatively large polydispersity index values indicated that the dispersions were considerably polydisperse, and the Z_{ave} sizes could not have high confidence. This is a well-known limitation of the dynamic light scattering technique, but

Table 1. Verification and calibration curve values obtained by ultraviolet-visible absorbance for multiwalled carbon nanotube dispersions

	CNT-N absorbance (535 nm)		CNT-A absorbance (537 nm)	
Concentration (mg/L)	Calibration value	Verification value	Calibration value	Verification value
2.5	0.252	0.151	0.283	0.133
5	0.344	0.167	0.567	0.227
10	0.749	0.235	1.104	0.448

CNT = carbon nanotube.

Table 2. *Z*-average diameter and polydispersity index obtained for multiwalled carbon nanotube dispersions

	CNT-N		CNT-A	
Concentration (mg/L)	$Z_{ave,mean}$ (nm)	PDI_{mean} (a.u.)	$Z_{ave,mean}$ (nm)	PDI_{mean} (a.u.)
Calibration dispersions				
2.5	255.5	0.383	325.7	0.454
5	269.5	0.380	348.8	0.447
10	354.6	0.480	332.7	0.485
30	297.9	0.477	364.4	0.469
Verification dispersions				
2.5	523.2	0.454	672.8	0.516
5	678.1	0.604	483.3	0.665
10	830.2	0.655	459.1	0.612

CNT = carbon nanotube; a.u. = arbitrary units; $Z_{ave,mean}$ = mean average *Z* diameter; PDI = polydispersity index.

these values were used only for comparative purposes of dispersion quality.

Furthermore, dynamic light scattering measurements showed that the sonication parameters selected (time and amplitude) for calibration curve dispersions were appropriate, with Z_{ave} diameters oscillating between 255.5 nm and 354.6 nm for CNT-N and 325.7 nm and 364.4 nm for CNT-A. Polydispersity indexes were in all cases lower than 0.5. Nevertheless, the data obtained for verification dispersions were less acceptable, with Z_{ave} diameters between 523.2 nm and 830.2 nm for CNT-N and 459.1 nm and 672.8 nm for CNT-A. Polydispersity indexes were also higher for these dispersions, exceeding in most cases 0.6. Moreover, differences in Z_{ave} diameters and polydispersity index for both types of CNTs were observed, as a result of their different physical properties (*Materials and Methods*; Supplemental Data, Table S1). The concentrations of dispersions analyzed involved also variations in dynamic light scattering parameters measured, with the lowest concentrations producing the most reduced Z_{ave} and polydispersity index.

Selection of the dispersion method and ecotoxicity tests

As mentioned in *Introduction*, sonication may modify attributes such as length of nanotubes and, hence, toxicity. Nevertheless, the amount of energy delivered by the sonicators was optimized to avoid damaging and cutting of CNTs, considering a previous study which calculated the minimum energy required to disperse an optimum amount of MWCNTs. On the other hand, the characterization performed suggested that the ultrasonic probe produced lower size distributions and rates of agglomeration than the ultrasonic bath, which demonstrated that the former produced a better optimization of the energy. This occurred despite the fact that the energy delivered by the ultrasonic bath (26.20 kJ) was slightly higher than the energy delivered by the ultrasonic probe (25.35 kJ). Therefore, the sonicator probe was the technique selected to prepare dispersions for ecotoxicity assessment.

With respect to the initial CNT concentrations for these dispersions, it was experimentally observed that the culture medium salt for bacteria (NaCl) reduced their stability. Furthermore, the photometer used to measure the luminescence of bacteria presents limitations for samples with light-absorbent colorants (the case of CNTs) because they can distort the results (the equipment corrects the absorbed light automatically, providing that the absorbances are below 1.800). These drawbacks were overcome by reducing the initial concentrations of MWCNTs to 10 mg/L (and the respective concentration of humic acid to 33.3 mg/L).

The size distribution graphs of the dispersions for ecotoxicity assessment (Supplemental Data, Figure S6) indicated a majority of MWCNTs forming larger agglomerates than in the case of dispersions prepared previously for calibration curves and verification. Ecotoxicity dispersions of CNT-N showed Z_{ave} diameters of 1654 nm with a polydispersity index of 0.460, whereas the same values obtained for CNT-A dispersions were 1048 nm and 0.437, respectively. Nevertheless, this fact was reasonable, considering the differences in the dispersion introduced by the culture medium. Moreover, the agglomerate size was acceptable because previous work on this issue reported similar or even greater Z_{ave} diameters [36,37]. Substantial differences were observed between the size distributions of the CNTs studied, caused by their different physical properties (see *Materials and Methods* and Supplemental Data, Table S1). These divergences were consistent with those observed in the previous subsection because, in the case of 10 mg/L dispersions, Z_{ave} diameters and polydispersity indexes of CNT-N dispersions were higher than those of CNT-A.

The dispersions for ecotoxicity assessment were also characterized by UV-visible spectroscopy to check whether concentrations of MWCNTs (10 mg/L) produced the expected absorbances according to calibration curve values. The data were slightly higher (3.7% for CNT-N and 2.8 % for CNT-A) than those of calibration curves, probably because of the variations introduced in the sonication process (initial concentrations) and the differences in the agglomerate size. However, if these absorbance values are represented in the calibration curves, the linear fits between the measured absorbances and concentrations remain, with $r^2 > 0.990$. Thus, these divergences were acceptable and the calibration curves obtained by UV-visible absorbance were useful to measure CNT concentrations in ecotoxicity dispersions.

Independent ecotoxicity tests on *V. fischeri* bacteria were carried out with humic acid to check that the concentrations used were nontoxic and did not alter the toxicity results of CNTs. Values provided in Table 3 experimentally demonstrated that concentrations of 100 mg/L humic acid did not cause inhibition. Moreover, solutions with higher concentrations (300 mg/L) were tested with the aim of obtaining the EC50 and EC20 endpoints of humic acid.

Finally, ecotoxicity tests with *V. fischeri* bacteria were conducted. Table 4 shows the ecotoxicity results obtained for the MWCNTs studied.

The reported data of CNT toxicity on luminescent bacteria are limited to a few studies, as mentioned in *Materials and Methods* [33,34]; and, otherwise, these data are divergent. The

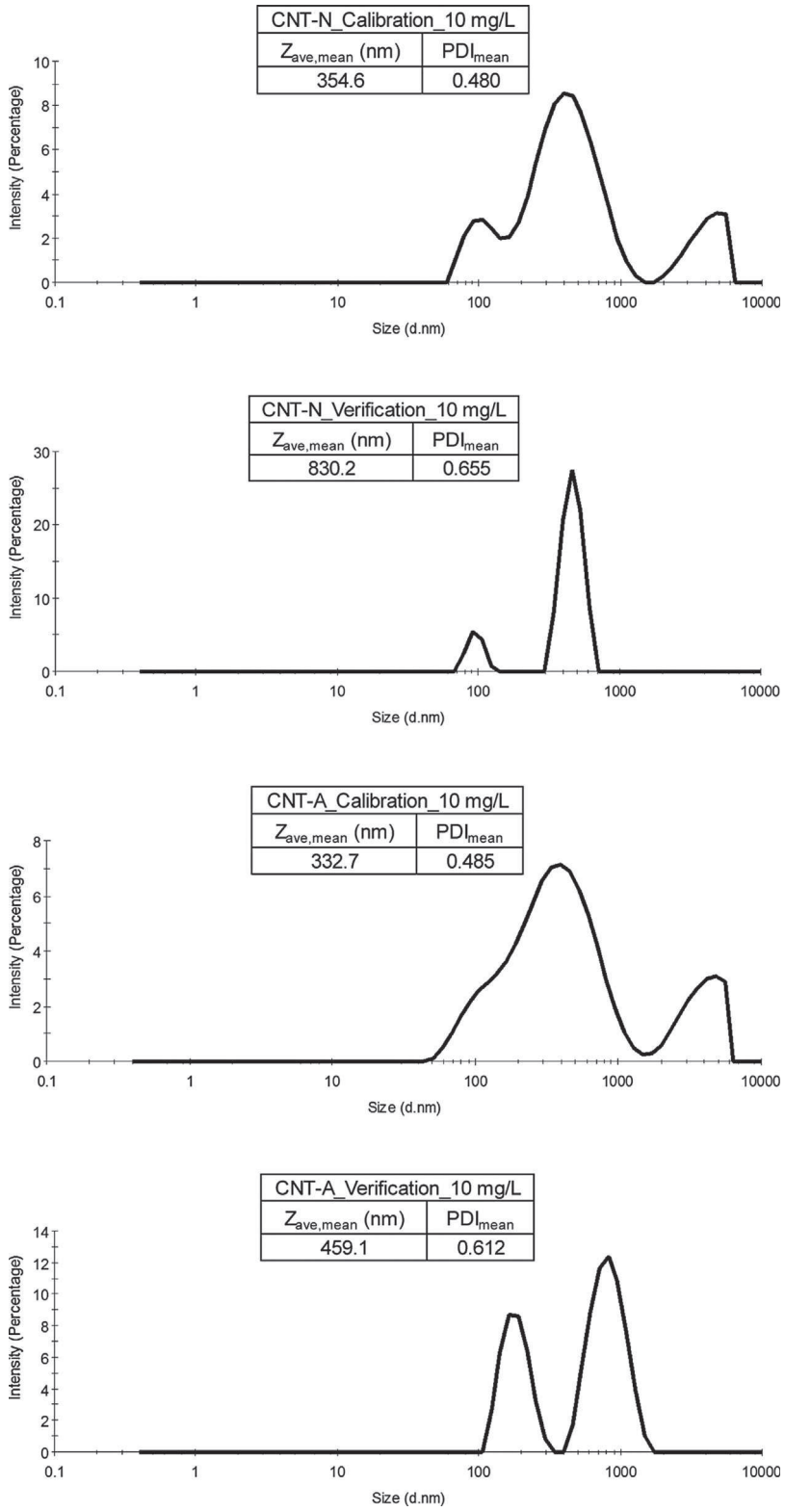

Figure 5. Size distributions by intensity of carbon nanotube agglomerates in 10 mg/L calibration and verification dispersions. CNT = carbon nanotube; $Z_{ave,\ mean}$ = mean average Z diameter; PDI = polydispersity index.

literature has demonstrated that SWCNTs are more toxic than MWCNTs to bacteria and microbial communities of aquatic systems [38,39]. However, the EC50 for SWCNTs on *V. fischeri* after an exposure time of 15 min has been reported to be higher than 100 mg/L [33], although this endpoint ranged between 50 mg/L and 84 mg/L in the case of MWCNTs in another study [34]. Thus, a systematic understanding of their real toxicity is required. Regarding the toxicity results obtained in the present study for MWCNTs, lower values for the EC50 endpoint were observed with respect to the data mentioned [34]. Those divergences are reasonable, given that the physical properties of CNTs studied and the exposure duration in that case (limited to 15 min) were different. In addition, the toxicity of MWCNTs to *V. fischeri* has been reported to be related to the

Table 3. Toxicity (30-min EC50 and EC20) of humic acid solutions on bacteria *Vibrio fischeri* (mg/L)

Sample	EC50 ± SD[a]	EC20 ± SD[a]
Humic acid-100[b]	>50	>50
Humic acid-300[b]	245.0 ± 2.7	165.7 ± 9.4

[a]Standard deviation of tests conducted in triplicate.
[b]Humic acid-100 and Humic acid-300 indicate humic acid concentration (milligrams per liter) of test samples. The dilution series were prepared from these initial concentrations in order to perform the complete test.
EC20, EC50 = toxicant effective concentrations causing 20% and 50% inhibition in light emission, respectively; SD = standard deviation.

Table 4. Toxicity (30-min EC50 and EC20) of multiwalled carbon nanotube dispersions on bacteria *Vibrio fischeri* (mg/L)

Sample	EC50 ± SD[a]	EC20 ± SD[a]
CNT-N/humic acid	5.4 ± 1.3	1.8 ± 0.6
CNT-A/humic acid	6.8 ± 2.1	2.0 ± 0.3
Humic acid	>16.7	>16.7
$K_2Cr_2O_7$	19.4 ± 3.5[b]	2.0 ± 0.7[b]

[a]Standard deviation of tests conducted in triplicate.
[b]The validity criteria for the acceptance of the test results were fulfilled since a concentration of 11.3 mg/L $K_2Cr_2O_7$ produced between 20% and 80% inhibition after 30 min of exposure.
CNT = carbon nanotube; EC20, EC50 = toxicant effective concentrations causing 20% and 50% inhibition in light emission, respectively; SD = standard deviation.

tube size, with the smallest diameters showing greater toxicity [34,38,40]. The influence of size was also observed in the present study because CNT-N nanotubes had lower diameter and length than CNT-A and the concentration required to produce 50% inhibition in light emission of bacteria was smaller than in the case of CNT-A.

The ecotoxicity results depend on the properties of the CNTs and the parameters used in the test, such as exposure time. Hence, standardization of the methodologies to assess their toxic effects is necessary to obtain comparable results between different studies. The present study represents an important contribution to the selection of the most appropriate dispersion methods, which is a key step in the process of standardization. Thus, the ecotoxicity data obtained should be considered in terms of reliability.

CONCLUSIONS

The present study has demonstrated that UV-visible absorbance absolutely depends on the sonication method. Therefore, dispersions for calibration curves and for toxicity assessment should be prepared using the same method to achieve the same absorbance results. Moreover, a new procedure to select the most appropriate measurement wavelength for each type of MWCNT has been proposed. The experimental data obtained in the present study have permitted optimization of the parameters selected to prepare dispersions for conducting an ecotoxicity assessment of MWCNTs on *V. fischeri* bacteria. Considering the lack of standardization in the study of the environmental effects of MWCNTs to date and the few and divergent available data for their toxicity on *V. fischeri*, the reliability of the present study's results should be taken into account.

SUPPLEMENTAL DATA

Tables S1–S2.
Figures S1–S6. (146 KB DOC).

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Data availability—The majority of data, associated metadata, and calculation tools are available through the Supplemental Data. The rest of the information is available on request (cristina.cerrillo@tekniker.es).

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SUPPLEMENTAL DATA

Ecotoxicity of multiwalled carbon nanotubes: standardization of the dispersion methods and concentration measurements

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MATERIALS AND METHODS

Materials for dispersions

The commercial MWCNTs used were Nanocyl NC7000 (Nanocyl), referred as CNT-N, and Arkema Graphistrength C100 (Arkema), referred as CNT-A. Their physical descriptions were provided by the manufacturers and are specified in Table S1.

Table S1. Physical descriptions of MWCNTs studied

MWCNT type	Description	Outer diameter (nm)	Inner diameter (nm)	Length (nm)	Purity (%)	Impurities (metal oxides) (%)	Surface Area (m^2/g)
Nanocyl NC7000 (CNT-N)	CCVD multiwall carbon nanotubes	6-24	2-9	2000-5000	>95	<5	250-300
Arkema Graphistrength C100 (CNT-A)	CCVD multiwall carbon nanotubes	10-15	-	100-10000	>90	<10	-

CCVD = Catalytic chemical vapor deposition.

Dispersions preparation and characterization

Calculation of the total amount of energy delivered by the sonication methods from calorimetry. The delivered acoustic energy supplied by the ultrasonicators was calculated using the calorimetric method described by Taurozzi et al [1].

The acoustic powers delivered by sonicator probe and bath were calculated in a similar manner. A 600 mL borosilicate glass beaker was filled with 500 mL thermally equilibrated MilliQ water. Its temperature and mass were measured with an uncertainty of ±0.1 ºC and ±0.1 g, respectively. In the case of the ultrasonic probe, the 600 mL beaker was placed in the sonicator chamber and the tip was immersed to a position 2.5 cm below the liquid surface. The temperature probe was mounted (using a clamp) at 2.5 cm depth and 1 cm away from the

sonicator probe. The sonicator output selected was 60% amplitude, operating in continuous mode. The temperature increase of the water was recorded for 5 minutes with a time resolution of 15 seconds. Sonicator bath was filled with deionized water at the same level as the one inside the 600 mL beaker and the temperature probe was mounted (using a clamp) at 2.5 cm depth in the center of the beaker. The sonicator operated in continuous mode and the water temperature increase was recorded for 60 minutes with a time-resolution of 2 minutes.

Calculation of the delivered acoustic energy was performed obtaining the best linear fit (R^2>0.990) between the measured temperature and time using least squares regression. The effective delivered power was determined using the Equation S1

— (S1)

where P is the delivered acoustic power (W), dT/dt is the slope of the regression curve, M is the mass of liquid (g), and C_p is the specific heat of the liquid ($J \cdot g^{-1} \cdot {}^oC^{-1}$).

The effective delivered acoustic power (P) was 42.26 W for sonicator probe and 7.28 W for sonicator bath. The linear fits between the measured temperature as function of time using least squares regression are represented in Figure S1.

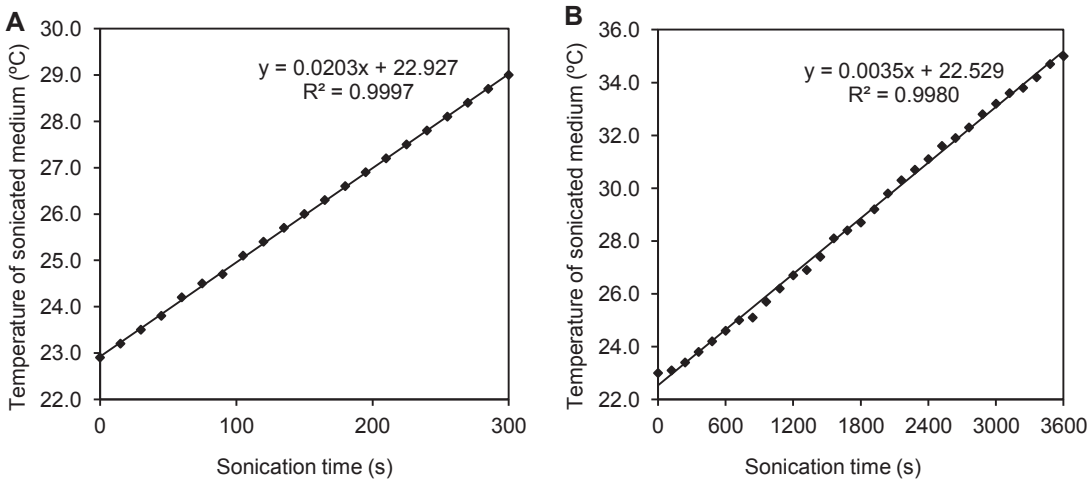

Figure S1. Linear fits between the measured temperature as function of time sonicator probe (**A**) and bath (**B**)

The total amount of energy delivered (Eqn. S2) was obtained considering the applied power and also the total amount of time that the water is subjected to the ultrasonic treatment

45 (S2)

46 where E is the total amount of energy (J), P is the delivered acoustic power (W) and t is the
47 total amount of time (s).

48 It is important to consider that the actual volumes and temperatures of MWCNTs dispersions
49 were different from that used in the calculation of the energy delivered by the sonication
50 methods. This aspect is noted in the calorimetric method, which is simply intended to allow the
51 reporting and transference of sonication power levels between users, but not to measure the
52 actual fraction of power utilized for powder disruption under specific dispersion conditions.

53 **RESULTS AND DISCUSSION**

54 *Calibration curves and verification*

55 Table S2. UV/vis absorbance values to perform calibration curves, for each dilution level and type of MWCNT

Concentration (mg/L)	CNT-N Absorbance (535 nm)	CNT-A Absorbance (537 nm)
30	-	-
25	1.754	-
20	1.440	-
15	1.045	1.685
10	0.749	1.104
5	0.344	0.567
2.5	0.252	0.283
2	0.153	0.236
1.5	0.107	0.186
1	0.080	0.126
0.5	0.039	0.071
0.25	0.020	0.040
0.1	0.000	0.015

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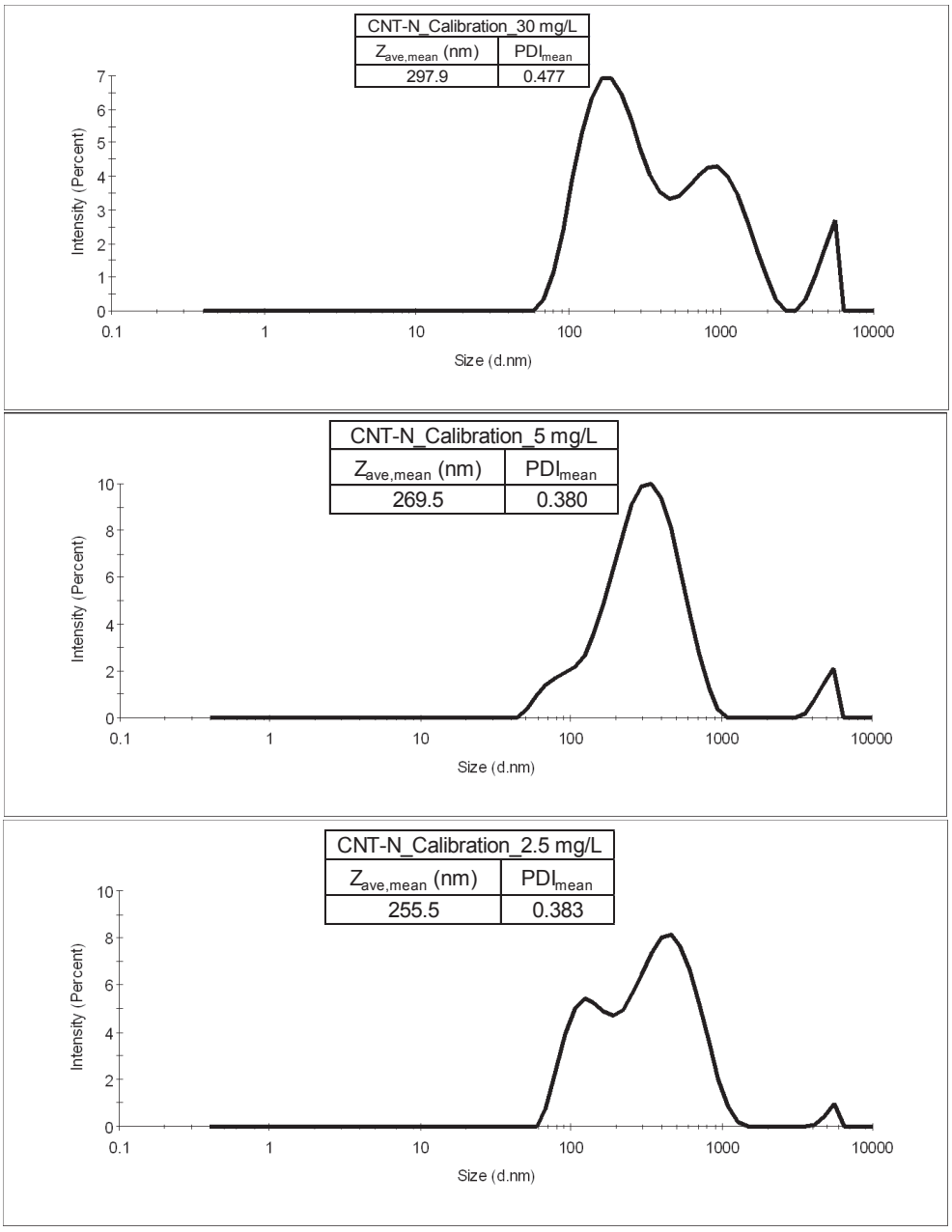
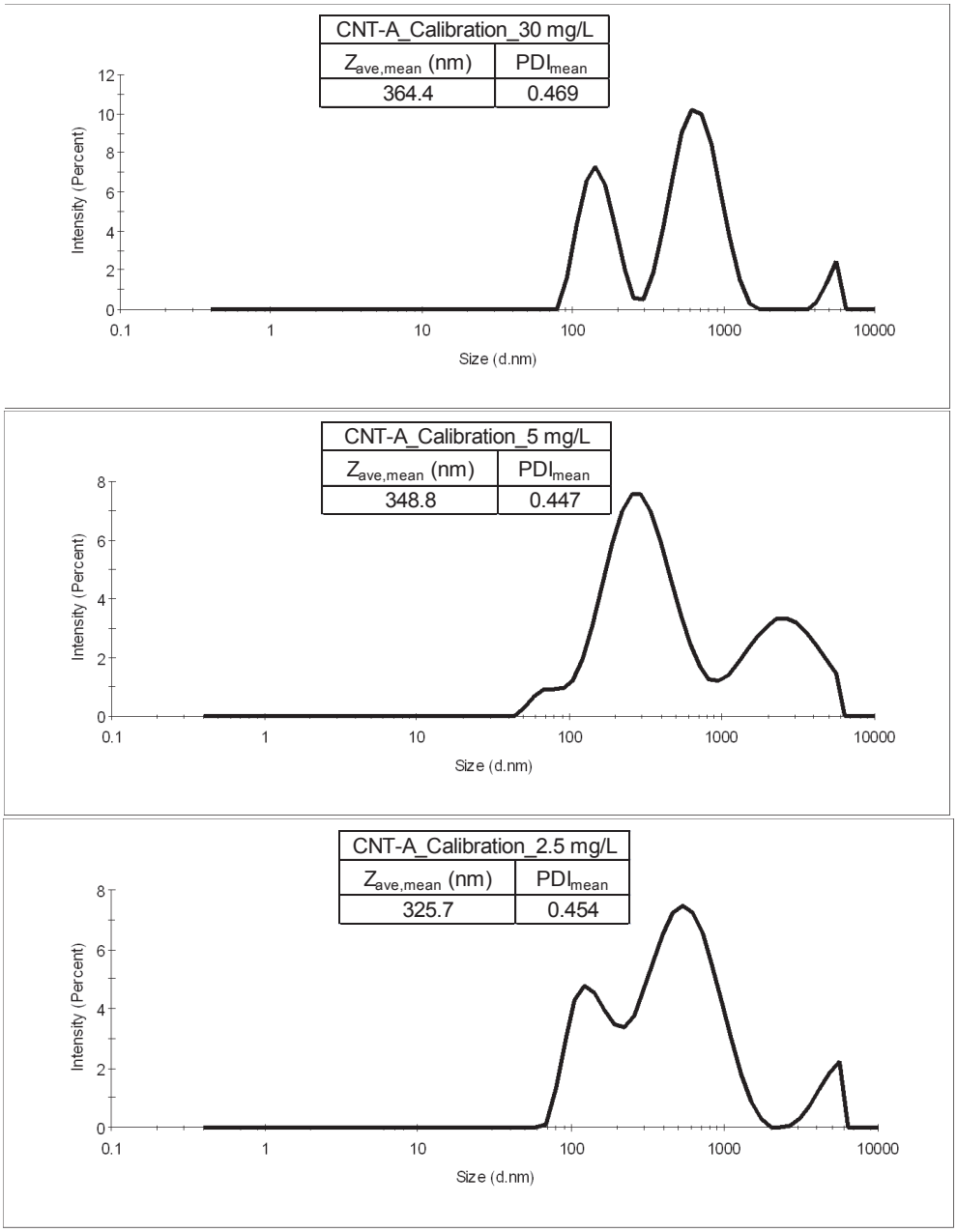
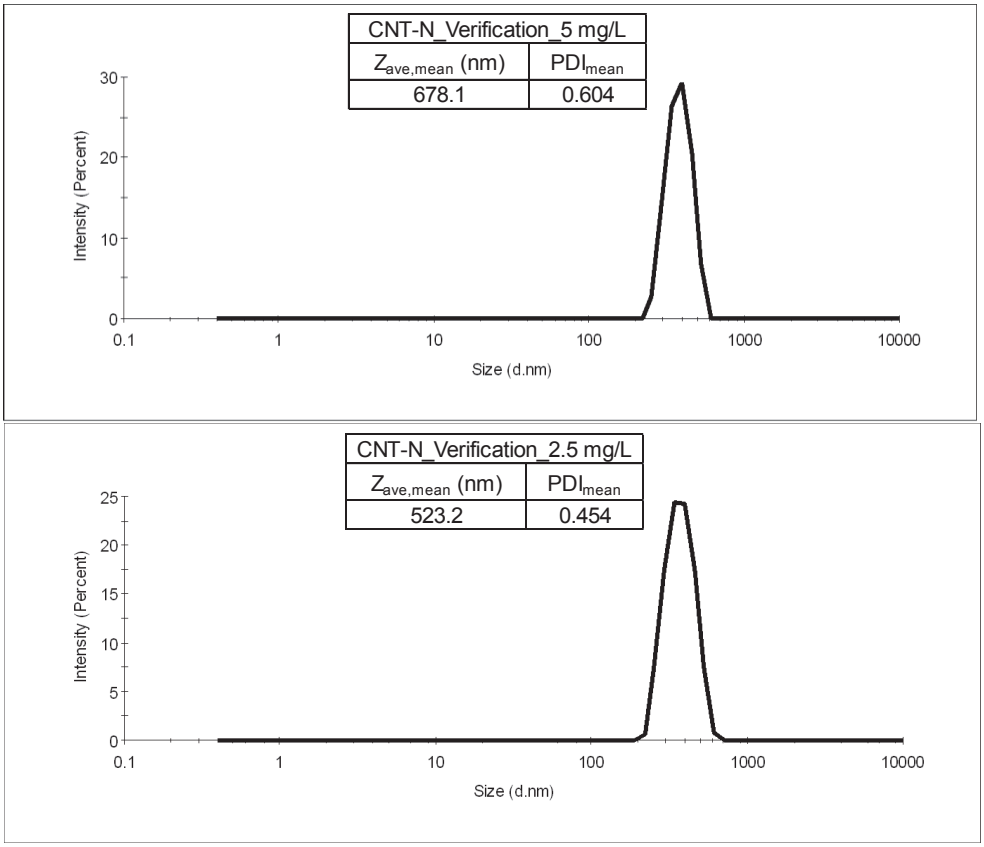
61 *Dispersions characterization by DLS*



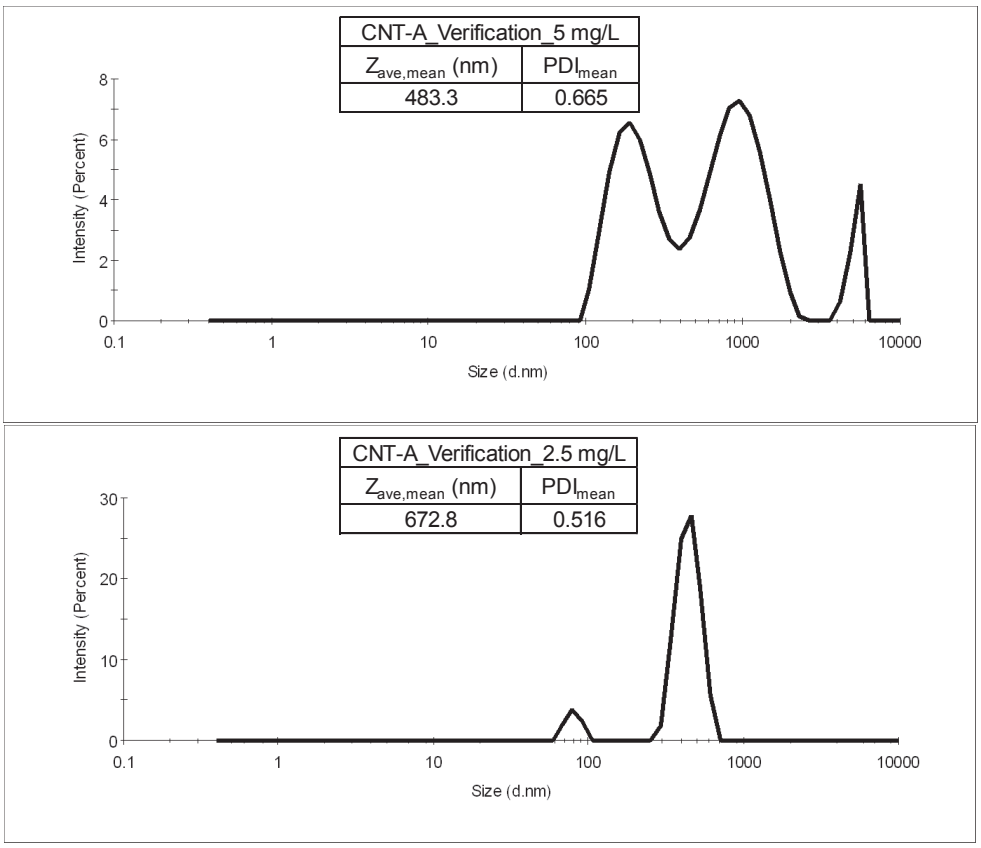
62 Figure S2. Size distributions by intensity of CNT-N nanotubes agglomerates in calibration dispersions.



63 Figure S3. Size distributions by intensity of CNT-A nanotubes agglomerates in calibration dispersions.



64 Figure S4. Size distributions by intensity of CNT-N nanotubes agglomerates in verification dispersions.



65 Figure S5. Size distributions by intensity of CNT-A nanotubes agglomerates in verification dispersions.

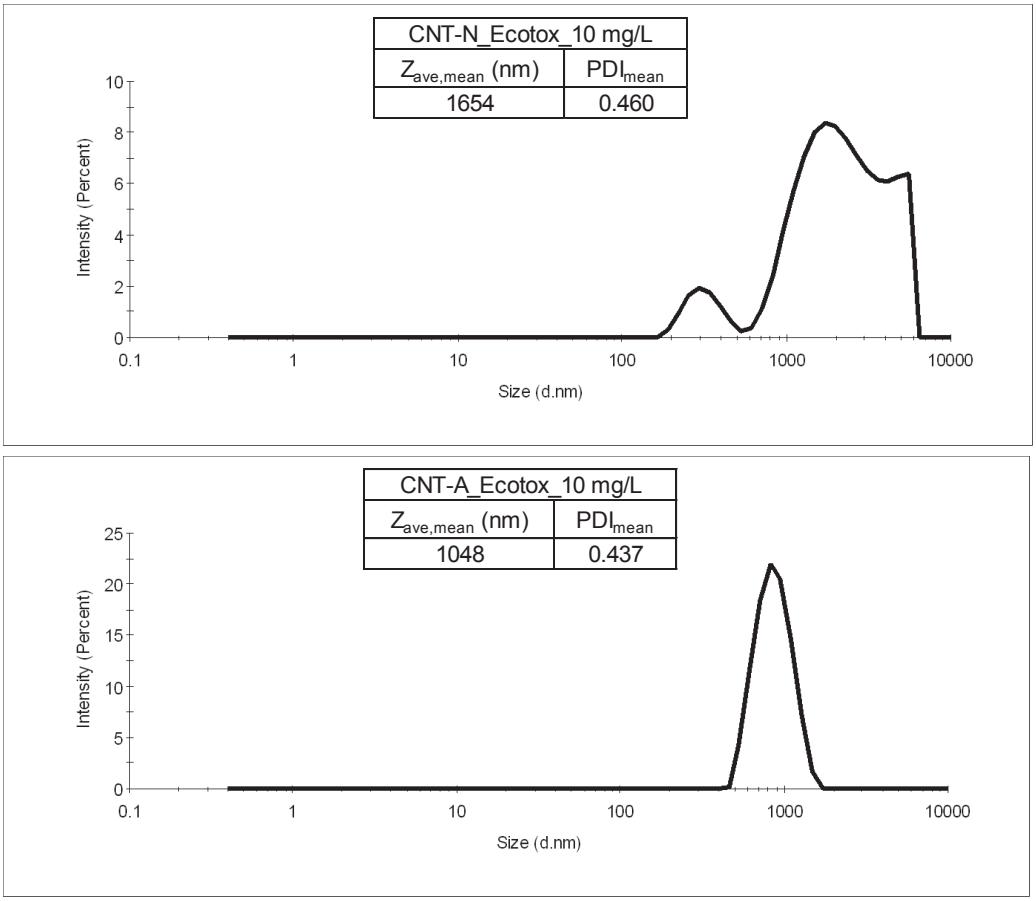


Figure S6. Size distributions by intensity of MWCNTs dispersions in HA and *Vibrio fischeri* medium for ecotoxicity assessment.

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COLLOIDAL STABILITY AND ECOTOXICITY OF MULTIWALLED CARBON NANOTUBES: INFLUENCE OF SELECT ORGANIC MATTERS

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Abstract: In the last few years, the release of multiwalled carbon nanotubes (MWCNTs) into the environment has raised serious concerns regarding their fate and potential impacts. Aquatic organisms constitute an important pathway for their entrance and transfer throughout the food web, and the current demand for standardization of methodologies to analyze the interactions of MWCNTs with them requires aquatic media that represent natural systems. However, the inherent hydrophobicity of MWCNTs and the substances present in natural waters may greatly affect their stability and bioavailability. The present study analyzes the influence of the most referenced synthetic and natural organic matters (Sigma-Aldrich humic acid and Suwannee River natural organic matter) in the agglomeration kinetics and ecotoxicity of MWCNTs, with the aim of determining their suitability to fulfill the current standardization requirements. Natural organic matter provides increased colloidal stability to the MWCNTs' dispersions, which results in higher adverse effects on the key invertebrate organism *Daphnia magna*. Furthermore, the results obtained with this type of organic matter allow for observation of the important role of the outer diameter and content impurities of MWCNTs in their stability and ecotoxicity on daphnids. Sigma-Aldrich humic acid appeared to alter the response of the organisms to carbon nanotubes compared with that observed in the presence of natural organic matter. *Environ Toxicol Chem* 2015;9999:1–10. © 2015 SETAC

Keywords: Ecotoxicity Multiwalled carbon nanotubes Organic matter Dynamic light scattering Sonication

INTRODUCTION

Carbon nanotubes (CNTs) exhibit extraordinary physicochemical properties that are useful in many applications in different fields such as chemistry, electronics, energy, materials science, and medicine [1,2]. As a consequence, their large-scale production is increasing, and considerable concerns exist regarding their release into the environment and human exposure [3–5]. The number of industrial facilities for the relatively low-cost production of multiwalled carbon nanotubes (MWCNTs) is experiencing rapid growth compared with that of single-walled carbon nanotubes [6], and thus a greater release of the former is expected. However, divergent results for the toxicity of MWCNTs have been published [4,7], and this limited understanding of their environmental, health, and safety aspects poses a threat to their potential applications. These inconsistencies are originated by factors such as impurities, surface modifications, variable structures of carbon nanotubes, and exposure routes [4].

Aquatic organisms represent one of the most important pathways for the entrance and transfer of MWCNTs throughout the food web in ecosystems [8,9]. Nevertheless, the inherent hydrophobicity of MWCNTs usually results in an agglomeration and settlement behavior, which hinders their stability for ecotoxicological assessment in aqueous systems. In addition, the solution chemistries of aquatic environments influence their stability and thus determine their bioavailability. Previous studies have analyzed the interactions between MWCNTs and the substances present in natural waters, such as monovalent and divalent salts as well as natural organic matter (NOM) [10–12]. High ionic strength and low pH induce the colloidal destabilization of MWCNTs, whereas humic substances (the major fraction in NOM) promote their stabilization [13,14]. The adsorption of NOM by MWCNTs also has been studied for separation and purification applications for drinking water [15,16].

Humic acid and fulvic acid are the components of humic substances distributed in aquatic environments [17]. Humic acid is the main fraction of NOM and exhibits a higher molecular weight than fulvic acid [18]. It has been widely used in ecotoxicity assessments of MWCNTs, especially in its commercially available form synthesized by Sigma-Aldrich [12,19,20]. Furthermore, Suwannee River NOM and humic acid (SR-NOM and SR-humic acid, respectively) [17] are the most extensively used natural organic substances to study the bioavailability of MWCNTs [10,11,14,21,22]. Their key advantage is the simulation of the real ecosystems in the toxicity assays, unlike laboratory-synthesized humic substances. Natural organic matter is a more representative sample than natural humic acid of what is found naturally, composed of chemically complex polyelectrolytes with varying molecular weights, and produced mainly from the decomposition of plant and animal residues [11].

The type of organic matter used in the tests has been shown to influence the adverse effects of MWCNTs on aquatic organisms [23,24]. Previous studies have compared the behavior of MWCNTs in the presence of organic matter from different sources, namely, Sigma-Aldrich humic acid and soil loam [12], NOM and humic substances [11,16,24], and different humic acids [13,25–27]. However, the understanding of the behavior of MWCNTs in the aquatic environment needs operational procedures that represent natural systems [28], and the current demand for standardization of materials and methods to analyze their ecotoxicity remains unsolved [29].

This article includes online-only Supplemental Data.

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The present study compares the agglomeration kinetics and ecotoxicity of MWCNTs in the presence of the most referenced synthetic and natural organic matters (Sigma-Aldrich humic acid and SR-NOM, respectively), with the aim of determining which is most appropriate to fulfill the current regulation requirements. The standardization approach of the present study also includes the calculation and optimization of the energy delivered to the MWCNTs during the preparation of dispersions. Some previous works did not consider this aspect [8,24], resulting in significant damage to the MWCNTs, which may alter their behavior within the context of toxicological testing [4,30]. Inhibitory effects on the key invertebrate organisms for regulatory testing *Daphnia magna* were studied, considering also that several experimental data on toxicity of MWCNTs toward them have been published [8,24,31–33].

MATERIALS AND METHODS

Materials for dispersions

Multiwalled carbon nanotubes were obtained from commercial sources and from the Joint Research Centre—European Commission Repository. All of them were produced via catalytic chemical vapor deposition. The MWCNT physical descriptions and impurity percentages were provided by the manufacturers (Table 1 and Supplemental Data, Table S1). Additional purification steps were not performed with MWCNTs to analyze the influence of the amounts of impurities on their toxic effects.

Standard SR-NOM obtained from the International Humic Substances Society was used as a model NOM. Humic acid, selected as a model synthetic organic matter, was purchased from Sigma-Aldrich. Both substances were used without any further purification.

All stock solutions and dispersions were prepared in ultrapure water, produced by a Milli-Q water filtration system (Millipore). The rest of chemicals used were p.a. grade and obtained from Sigma-Aldrich and Scharlab.

Preparation of MWCNT dispersions

To prepare the organic matter solutions, 20 mg humic acid/SR-NOM were added to 1 L of either culture medium of the organisms (see detailed preparation in the Supplemental Data) or ultrapure water alone, and mixed on a magnetic stirrer for 72 h at 20 ± 2 °C. This time was sufficient to achieve a complete dissolution of the organic matter. Hence, a subsequent step of centrifugation or filtration to extract the supernatants was not necessary. Humic acid and SR-NOM concentrations were the same, to obtain comparable results for the agglomeration kinetics and ecotoxicity of MWCNTs. They were selected by taking into account the amounts in natural waters [20,34,35].

The dispersions were obtained by adding 10 mL of humic acid/SR-NOM solution to 0.5 mg MWCNTs in 20-mL glass scintillation vials and then sonicated with an ultrasonic homogenizer, a widely accepted method that ensures reasonable stability [13,24]. The MWCNT concentrations were selected according to the short-term endpoints reported in the literature for *D. magna* tests [3,31,33]. The ultrasonic homogenizer (VIBRACELL-VCX750, SONICS&MATERIALS) operated with a standard probe (136-mm length and 13-mm diameter), at a frequency of 20 kHz, continuous mode for 16 min and output power fixed at 750 W at 40% amplitude. The calorimetric method described by Taurozzi et al. [30] was used to calculate the sonication time and amplitude required to optimize the acoustic energy delivered by the probe. Considering the previously reported energy required to achieve the maximum degree of dispersion of MWCNTs in aqueous solution without damaging CNTs [36], we established that the total amount of energy supplied to the dispersions should not exceed 30 KJ. Additional details of these calculations are provided in the Supplemental Data, Figure S1. During sonication, the vials were held in an ice bath to minimize temperature rising of the sample, and the probe was inserted between the upper quarter and upper half of the dispersion volume in the vials. These conditions were essential to maximize the liquid-probe surface area exposed to the acoustic waves, as well as the container wall surface to volume ratio for dissipation of heat by the cooling bath [30].

The dispersions were characterized and tested immediately after their preparation. Subsequent steps of settling or centrifugation were avoided, because potential changes such as agglomeration and sedimentation were considered reactions occurring in the test systems.

Table 1. Physical descriptions of the multiwalled carbon nanotubes studied

Code	Outer diameter (nm)	Length (nm)
CNT-1	6–24	2000–5000
CNT-2	10–15	1000–10000
CNT-3	9–18	400–1300
CNT-4	28–99	1600–6500

CNT = carbon nanotube.

Characterization of MWCNT dispersions

Dynamic light scattering, ultraviolet–visible (UV/Vis) spectroscopy, and scanning electron microscopy (SEM) characterization were conducted in all the dispersions immediately after their preparation and at the end of the ecotoxicity tests. A slight shaking for homogenization preceded the characterization of the dispersions at the end of the tests. Sampling the aquatic phase would have required additional settling or centrifugation steps, because sedimented or agglomerated MWCNTs were not clearly observed.

The stability of the dispersions was assessed by measuring the variation in scattered light intensity and calculated average zeta-sizes as a function of time. For this purpose, Zeta-average diameter (Z_{ave}) and polydispersity index values were obtained by dynamic light scattering measurements in a Malvern Zetasizer Nano ZS instrument, considering the data generated from 10 repeated measurements.

Ultraviolet–visible spectroscopy was used to determine quantitatively MWCNT concentration in dispersions and to study the influence of humic acid and SR-NOM in the agglomeration kinetics of MWCNTs. This is one of the most reported techniques in the last 10 yr to determine concentrations, given its rapidness and low cost [37,38]. The absorbances of dispersions with previously known concentrations were measured to obtain the corresponding calibration curves. These calibration dispersions were prepared with humic acid/SR-NOM dissolved in ultrapure water (without adding culture medium), with starting concentrations of 50 mg/L MWCNTs. Dilution levels, as well as the UV/Vis absorbance results of the calibration dispersions, can be found in the Supplemental Data, Figures S2 and S3. The apparent concentrations of MWCNTs in batch dispersions (prepared with humic acid/SR-NOM dissolved in culture medium) were obtained from the UV/Vis absorbances of the calibration dispersions. All of the measurements were conducted immediately after sonication processes at 530 nm, using an

ultraviolet-visible-near infrared (UV/Vis/NIR) spectrophotometer (Lambda 950, PerkinElmer) and quartz cells with 10-mm path length. The selection of the wavelength was carried out considering that previously reported for MWCNTs [39]. Although the absorbance peaks of the nanotubes studied were observed at lower wavelengths, saturation of the spectrophotometer was reached in this region of the spectrum. Moreover, the absorbance peaks of organic matter occur at lower wavelengths [21,40] and might have interfered with those of CNTs. The absorbance values of humic acid and SR-NOM were negligible at 530 nm and did not alter those obtained for MWCNTs. Nonetheless, the measurements were carried out considering humic acid and SR-NOM as background substances and subtracting their absorbance by the "autozero" function of the spectrophotometer. The absorbance of the nutrients in the culture medium was also subtracted for batch dispersion measurements, although their absorbance spectrum between 400 nm and 1200 nm was observed to be negligible.

Furthermore, SEM imaging was performed to support the results obtained by the previous characterization, using a Zeiss apparatus (ULTRA PLUS model). A drying process (24 h under ambient temperature) prepared the SEM samples. During this period, MWCNTs possibly formed larger agglomerates, and organic matter and culture media substances may have crystallized. Thus, this ultimate disposition was not totally comparable with what happened when they were in dispersion but showed the appearance of nanotubes after sonication and ecotoxicity tests.

D. magna acute immobilization tests

Neonates of *D. magna* used (aged less than 24 h) were obtained from Microbiotests. The assays were performed following the prescriptions of the Organisation for Economic Co-operation and Development *Daphnia* sp., acute immobilization test (guideline 202) [41] and the specifications included in the Supplemental Data. Each test involved 5 concentrations starting at 50 mg/L, and each concentration used 20 neonates (distributed in 4 replicates). The total number of dead and immobile neonates during exposure for 48 h was calculated for each dilution, and the concentrations bringing 20% and 50% immobilization (EC20 and EC50, respectively) as well as their associated 95% confidence limits were determined by regression analysis in Excel 2007 (Microsoft).

Because an essential aim of the present study was the assessment of the influence of the type of organic matter on the ecotoxicity of MWCNTs, the concentration of nutrients and pHs of the media were adjusted to the specific requirements for the test organisms. The initial pH values of culture medium and test dispersions were adjusted to 8.2 to 8.3, as high as possible considering the previously mentioned fact that low pH induces the colloidal destabilization of CNTs [13]. To check the validity of the test procedures, additional tests were carried out with the reference chemical potassium dichromate ($K_2Cr_2O_7$). The SR-NOM and humic acid solutions were also independently analyzed to verify that the concentrations used did not induce toxic responses.

RESULTS AND DISCUSSION

Characterization of MWCNT dispersions

Characterization by dynamic light scattering. A previous characterization by dynamic light scattering was performed with the 50 mg/L MWCNT calibration dispersions (Table 2 and Supplemental Data, Figure S4).

With respect to the initial MWCNT concentrations for these dispersions, the addition of culture medium for the toxicity tests substantially increased the parameters measured with dynamic light scattering. Table 3 shows Z_{ave} and polydispersity index of dispersions prepared for ecotoxicity assessment, at the beginning and end of the tests.

Except for the results obtained for CNT-4, polydispersity index values were higher than 0.7. The calibration dispersions showed quite different polydispersity index values in all cases, and lower than 0.5 for most. Similarly, although the batch dispersions presented micrometric agglomerates, the Z_{ave} diameters obtained for the calibration dispersions were the lowest, ranging from 200 nm to 600 nm. The considerable Z_{ave} and polydispersity index values obtained for the batch dispersions pose certain limitations of the dynamic light scattering technique for the characterization of the nanotube agglomerates. Nevertheless, it can be used as a tool to compare the relative nanotube stability and agglomeration [12,42]. Dynamic light scattering results were useful to analyze the differences between the dispersions prepared with the 2 types of organic matter studied, and in any case SEM images and UV/Vis measurements provided additional data for characterization.

Table 2. Z-average diameters (Z_{ave}) and polydispersity indexes (PDI) of carbon nanotube agglomerates in calibration dispersions

	CNT-1		CNT-2		CNT-3		CNT-4	
	SR-NOM	HA	SR-NOM	HA	SR-NOM	HA	SR-NOM	HA
$Z_{ave,mean}$ (nm)	253.7	251.8	199.1	208.1	180.2	257.8	601.1	587.4
PDI_{mean}	0.405	0.572	0.426	0.415	0.499	0.436	0.280	0.394

CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

Table 3. Z-average diameters (Z_{ave}) and polydispersity indexes (PDI) of carbon nanotube agglomerates in batch dispersions at the beginning and end of the tests

		CNT-1		CNT-2		CNT-3		CNT-4	
		SR-NOM	HA	SR-NOM	HA	SR-NOM	HA	SR-NOM	HA
$Z_{ave,mean}$ (nm)	0 h	1384	1821	1760	1914	2272	1284	2034	2249
	48 h	3187	2805	1763	1845	2822	1639	1719	2073
PDI_{mean}	0 h	0.809	0.753	0.805	0.725	0.773	0.708	0.533	0.418
	48 h	1.000	1.000	0.822	0.731	0.835	0.813	0.483	0.382

CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

Conversely, the size of agglomerates and polydispersity index values in the batch dispersions could be reduced by decreasing the initial concentration of MWCNTs. Previous studies have reported that high concentrations in the dispersion process result in an increased nanoparticle collision frequency and also may induce agglomerate or aggregate formation as particles collide and coalesce [30,42]. However, sufficiently high initial concentrations, which led to inhibitory effects, were necessary to assess the ecotoxicity of the dispersions. Considering the previously reported average nanotube Z_{ave} sizes for ecotoxicity tests with *D. magna* [8,24], the results obtained in the present study were generally higher. As mentioned, these previous works did not consider the acoustic energy supplied by the ultrasonic probe during the preparation of dispersions. Significant damage to the MWCNTs was observed in both cases, even forming a high fraction of functional groups [8], which may alter their ecotoxicity. Average diameters ranging from 800 nm to more than 2 μm have been reported [33], more similar to those obtained in the present study. Furthermore, the settling of the dispersions after sonication to test only the supernatant is a common procedure to discard any undispersed MWCNTs and obtain better Z_{ave} and polydispersity index values [23,24], but it does not represent what takes place in natural environments. Agglomerated and sedimented nanotubes are expected to be very persistent [14]. Moreover, aquatic environments are not static media, and agglomerates also might be present in suspension in lakes and rivers. Thus, the approach in the present study constituted a better approximation to a realistic situation.

The type of organic matter used seemed to be related to variations in the stability of the dispersions. At the beginning of the tests, 3 of the MWCNTs analyzed showed lower Z_{ave} for SR-NOM dispersions. After 48 h of exposure, a clear trend was not observed in the agglomerate sizes, neither in the presence of SR-NOM nor in the presence of humic acid. However, in the case of the dispersions prepared for calibration curves without culture medium, SR-NOM produced a decrease in Z_{ave} or polydispersity index values for all of the nanotubes studied, suggesting that it provided an increased stability to the dispersions with respect to humic acid. The present study performed an evaluation of the dispersant capability of 2 types of organic matter for a single culture medium. The nutrient salts of the medium were a key aspect of MWCNTs stability; thus, their behavior in ultrapure water should be taken as a reference to achieve the harmonization of the methodologies.

Regarding the size distributions of the 4 MWCNTs studied, differences were observed between them, probably because of their different physical properties (see Table 1). Specifically, the MWCNTs with the lowest outer diameters (CNT-2 and CNT-3) showed smaller Z_{ave}, especially for the calibration dispersions. Moreover, CNT-4 nanotubes, with the largest initial outer diameters, resulted in the lowest polydispersity index values for the calibration and batch dispersions, and they formed agglomerates with similar sizes to those of the rest of MWCNTs in the batch dispersions. In addition, CNT-4 nanotubes did not show polydispersity index values increase during the ecotoxicity tests. These facts suggest that larger-diameters produce more stable dispersions, which may affect their toxic effects (see section *Results and Discussion*—D. magna *acute immobilization tests*), and is consistent with the results obtained by Lin et al. [13], who demonstrated that MWCNTs with smaller outer diameters had lower potential to be dispersed and stabilized in the presence of humic acids. The different lengths of the MWCNTs studied did not show a clear influence on the stability of the dispersions analyzed.

Concerning the variation of dynamic light scattering parameters throughout the duration of the tests, a clear trend was not observed. However, in the case of CNT-4 nanotubes a decrease in Z_{ave} was found after 48 h and their polydispersity index value remained constant. These results were consistent with those obtained at characterization by UV/Vis spectroscopy (as detailed below).

Characterization by UV/Vis spectroscopy

The fact that the agglomerates diameters influence the UV/Vis absorbances is generally accepted. If the agglomerate sizes are comparable to the light wavelength of the measurements, the intrinsic properties of MWCNTs are the main influencing factor on UV/Vis absorption. However, the agglomerate sizes are the main influencing factor if they are much larger than the wavelength, and poorly dispersed MWCNT agglomerates have a decreased apparent absorption coefficient [37]. The measurement wavelength in the present study was 530 nm, and agglomerates were quite a bit larger for batch dispersions. Therefore, Z_{ave} should be directly related to UV/Vis absorbances. However, the negative correlation expected between both parameters was only observed in the case of the calibration dispersions (Figure 1). The linear-least square fits were not represented, because lower agglomerate diameters did not result in increased absorbances in all cases, and the statistical spread was considerable.

The simulation of realistic environments involved that batch dispersions were greatly unstable, and the comparison of absorbance and agglomerate size measurements sometimes led to contradictory conclusions. This could be explained by the fact that the UV/Vis absorptions of the batch dispersions might be altered by the sonication of MWCNTs with the salts present in the culture medium. Furthermore, the exudates released by daphnids to mitigate the stress induced during the exposure period [43] might have absorbed in the peak wavelength range of carbon nanotubes, thus interfering with their UV/Vis absorption. Nevertheless, the UV/Vis results were meaningful to assess the dispersion capability of the organic matters used, and overall, SR-NOM produced better and more uniform absorbance results than humic acid, as observed for the dynamic

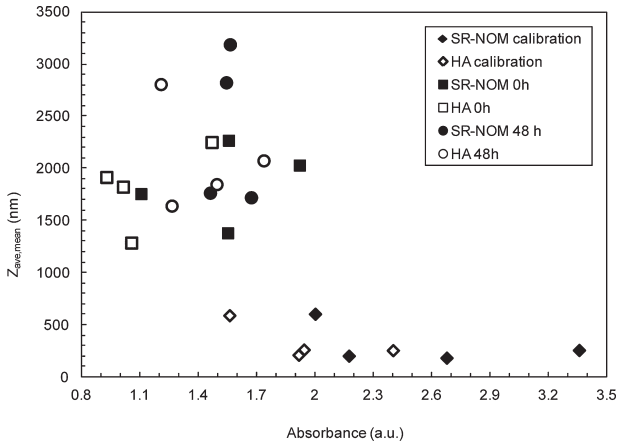

Figure 1. Correlation between Z_{ave} and absorbance results obtained for calibration and batch dispersions of MWCNTs. $Z_{ave,mean}$ = mean average Z diameter; a.u. = arbitrary unit; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

Table 4. Apparent multiwalled carbon nanotube concentrations in batch dispersions at the beginning and end of the ecotoxicity tests, obtained by ultraviolet–visible spectroscopy absorbance measurements

		CNT-1		CNT-2		CNT-3		CNT-4	
		SR-NOM	HA	SR-NOM	HA	SR-NOM	HA	SR-NOM	HA
Absorbance (a.u.)	0 h	1.5491	1.0135	1.1025	0.9278	1.5537	1.0560	1.9188	1.4699
	48 h	1.5621	1.2078	1.4616	1.4950	1.5443	1.2639	1.6727	1.7372
Apparent concentration (mg/L)	0 h	20.68	20.32	25.45	24.10	28.88	25.43	47.88	46.81
	48 h	20.85	24.35	33.78	39.03	28.70	30.68	41.71	55.38

CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

light scattering characterization. The same behavior was found for the dispersions prepared for calibration curves, in which higher values were obtained particularly for UV/Vis absorbances of SR-NOM dispersions (Figure 1 and Supplemental Data, Figures S2 and S3).

The apparent concentrations of MWCNTs in batch dispersions are shown in Table 4. Generally, they were lower than their corresponding nominal concentrations in calibration dispersions (50 mg/L), given the reduction in their stability promoted by the use of culture medium in their preparation. The apparent concentrations at the beginning of the tests were higher for SR-NOM. However, after 48 h, the opposite effect was observed, because the dispersions prepared with humic acid presented higher concentrations in all cases. Regarding the values obtained for the MWCNTs studied, the highest absorbances corresponded to CNT-4. This result was in accordance with dynamic light scattering characterization, because CNT-4 dispersions showed an increase in stability and the lowest polydispersity index. In the case of humic acid dispersion with CNT-4, a notable increase of absorbance occurred, corresponding to an apparent concentration even higher than the initial 50 mg/L.

Considering the behavior of dispersions throughout the duration of the tests, a general increase of MWCNTs absorbances was observed after 48 h for both SR-NOM and humic acid dispersions. As previously observed, this finding was consistent with the decrease in Z_{ave} found at the end of the tests for CNT-4. The accumulation and processing of MWCNTs by daphnids might alter the agglomeration state of the dispersions, and Edgington et al. [24] reported disaggregation of MWCNTs in the gut tract of *D. magna*. Conversely, the uptake of the nutrient salts by the organisms could pose a stabilization of MWCNTs during the test, as well as slight pH variations (see section *Results and Discussion*—D. magna *acute immobilization tests*).

Characterization by SEM

The imaging conducted (Figures 2, 3, 4 and Supplemental Data, Figure S5) suggests that SR-NOM and humic acid were adsorbed on the carbon nanotubes. The absorption of organic matter on MWCNTs has been previously demonstrated by means of several techniques [11,24]. We found in some of the images that the adsorption of SR-NOM on nanotubes was higher than that of humic acid, for both calibration and batch dispersions. Natural organic matter adsorbed onto the nanotubes surfaces was observed in Figures 2A, 2C, 2G, 3A, 4A, and 4C (indicated by white arrows), whereas only Figure 4G showed clearly the presence of humic acid. This fact could explain the trend observed by dynamic light scattering and UV/Vis characterization, which indicated lower agglomerate sizes and more stability and uniform results for SR-NOM.

The SEM images of calibration dispersions (Figure 2) showed a greater homogeneity than the batch dispersions (Figures 3 and 4). This enhanced homogeneity was observed, for instance, in the CNT-free areas present in Figure 2C, 2D, and 2E. Considering the high magnification of the imaging, these areas gave an indication of the presence of smaller agglomerates. These observations supported the quite lower polydispersity index values, Z_{ave}, and higher UV/Vis absorbance obtained in the previous characterization for the calibration dispersions.

The differences observed in dynamic light scattering and UV/Vis characterization between the types of nanotubes studied were supported by the SEM imaging. The MWCNTs with the lowest outer diameters for the bulk materials (CNT-2 and CNT-3) showed a decrease in Z_{ave}, which corresponded to MWCNTs better dispersed in SEM images, especially for the calibration dispersions (see Figure 2C, 2E). Moreover, the low polydispersity index values of CNT-4 were explained by a uniform dispersion of nanotubes observed in Figures 2G, 2H, and Supplemental Data, Figure S5. The fact that the agglomerates sizes of CNT-4 were similar to those of the rest of the MWCNTs for the batch dispersions was also observed in SEM imaging, considering their larger outer diameters and the lower magnification required to visualize them. The higher absorptions and apparent concentrations obtained with UV/Vis spectroscopy for CNT-4 were also in accordance with the homogeneous dispersions observed by SEM.

The decrease in Z_{ave} for CNT-4 and the overall increase in MWCNT apparent concentrations found after 48 h for both SR-NOM and humic acid batch dispersions were not clearly observed in SEM images (Figures 3 and 4; Supplemental Data, Figure S5). That SEM images can only show a tiny area of the samples and different agglomerate sizes in the same dispersion can be found is well known. Moreover, the preparation of SEM samples involves changes in the ultimate disposition of nanotubes.

Even though the batch dispersions prepared were greatly unstable, and the comparison between dynamic light scattering and UV/Vis spectroscopy measurements led to contradictory conclusions in some cases, SEM images supported the overall findings and insights obtained by the previous characterization.

D. magna acute immobilization tests

Given the variation of the apparent concentrations of MWCNTs in the batch dispersions observed during the tests, the initial concentrations of MWCNTs (50 mg/L) were selected to calculate EC20 and EC50. The dissolved oxygen measured in the controls and the batch dispersions was higher than 3 mg/L, in compliance with the validity criteria of the Organisation for

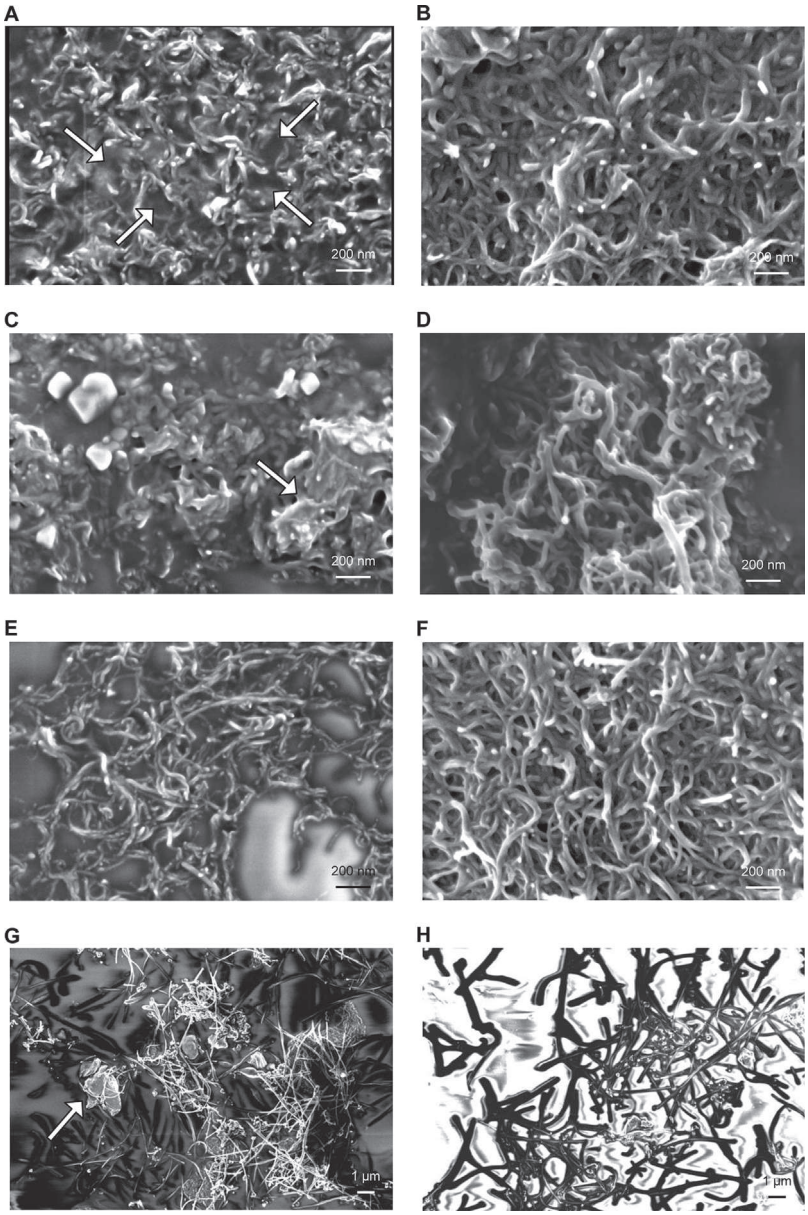

Figure 2. Scanning electron microscope images of the 50 mg/L multiwalled carbon nanotube calibration dispersions: (**A**) CNT-1- SR-NOM, (**B**) CNT-1-HA, (**C**) CNT-2-SR-NOM, (**D**) CNT-2-HA, (**E**) CNT-3-SR-NOM, (**F**) CNT-3-HA, (**G**) CNT-4-SR-NOM, (**H**) CNT-4-HA. CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

Economic Co-operation and Development guideline 202 [41]. The pH values at the end of the tests decreased slightly from the initial 8.3 to average values of 7.9, and they were kept in the range of the performance criteria. Two additional tests were carried out with the reference chemical $K_2Cr_2O_7$, and the EC50 values after 24 h of exposure were 1.11 mg/L and 0.96 mg/L, respectively (in the validation range of 0.6–2.1 mg/L). The 50% immobilization rates were not achieved for most of the dispersions tested. Thus, the EC20 values were used to analyze their effects on daphnids.

The results shown in Table 5 indicate that MWCNT dispersions prepared with SR-NOM exhibited greater toxicity levels than dispersions prepared with humic acid, taking into account that these 2 types of organic matter themselves did not cause inhibitory effects. This result was in accordance with the characterization conducted, which showed more stability for SR-NOM dispersions during the tests, and increased stability is assumed to lead to higher toxicological outcomes [24,31]. Specifically, for SR-NOM, the greater stability of the CNT-4 dispersions also contributed to confirm this assumption. The CNT-4s showed lower Z_{ave} with respect to the initial outer diameters, a decrease in the polydispersity index values, higher UV/Vis absorbance, and greater homogeneity in dispersions in the SEM images, compared with the rest of the MWCNTs. From a physicochemical perspective, the reason for the enhanced stability and increased toxicity with SR-NOM might be related to its nonhumic portion, which contains aliphatic carbon and nitrogen, including carboxylic acids, carbon hydrates, tannic acids, and proteins [44]. Wang et al. [27] reported that the key driving force for the sorption of NOM to MWCNTs were these alkyl (aliphatic) components rather than the aromatic ones of humic acid. Edgington et al. [24] also observed differences in the acute toxicity of MWCNTs to *D. magna*, depending on the sources of the NOM used, but they could not justify their results from either the suspensions or the NOM characterization. The nonhumic

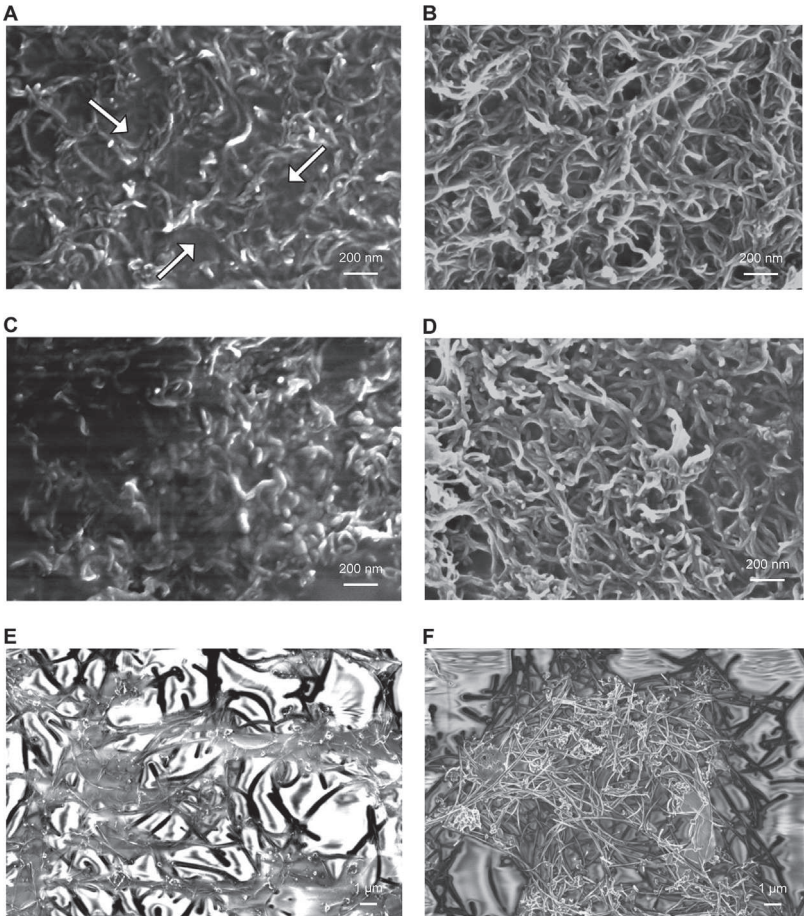

Figure 3. Scanning electron microscope images of the 50 mg/L multiwalled carbon nanotube batch dispersions at the beginning of the tests: (**A**) CNT-1-SR-NOM 0 h, (**B**) CNT-1-HA 0 h, (**C**) CNT-2-SR-NOM 0 h, (**D**) CNT-2-HA 0 h, (**E**) CNT-4-SR-NOM 0 h, (**F**) CNT-4-HA 0 h. CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

portion mentioned previously could be an influencing factor on the variations in acute toxicity that they obtained.

Furthermore, the current literature has reported the influence of the outer diameter, length, and rigidity of MWCNTs in their potential toxicity. Diameters of 50 nm have shown in vivo and in vitro effects, whereas thicker diameters (150 nm) or tangled (2–20 nm) are less toxic [4]. Conversely, the contribution of the amounts of metal impurities to the toxicity of MWCNTs also has been demonstrated [4,45]. The results obtained in the present study with SR-NOM were fully in accordance with CNT-4 (28–99 nm diameter) showing more adverse effects than the rest of the nanotubes studied (6–24 nm diameter). The CNT-4 nanotubes also had the highest content of impurities (Supplemental Data, Table S1). In the case of humic acid dispersions, CNT-4 did not produce toxic effects, which suggested that this type of synthetic organic matter might alter the response of organisms to MWCNTs with respect to that observed in the presence of SR-NOM. Toxicity also might be determined by a combined effect of the outer diameter and length of the carbon nanotubes. Liu et al. [4] and Lanone et al. [5] reported stronger adverse effects induced by the longest CNTs, which was consistent with the EC values and lengths provided in the present study. Considering that the outer diameters of CNT-1, CNT-2, and CNT-3 nanotubes were in similar ranges (Table 1), their toxic effects decreased with decreasing lengths in the presence of SR-NOM. Carbon nanotube-3, with lengths up to 1300 nm, showed the lowest toxicity; CNT-2, with lengths up to 10 000 nm, the highest. Carbon nanotube-1 presented intermediate lengths (up to 5000 nm) and EC values. Nonetheless, CNT-4 length was similar to that of CNT-1 and CNT-2, thus showing that the outer diameter of MWCNTs was a more decisive factor than length in determining the adverse effects on *D. magna*.

Regarding the previously reported endpoints for MWCNTs' ecotoxicity tests with *D. magna* [3,31,33], lower adverse effects were found in the present study because 48-h EC50 values were not achieved in most cases. This behavior was probably attributable to the fact that, in present study, a more realistic environment was reproduced in the preparation of the dispersions, which led to an increased instability and hence reduced toxicity. Moreover, the preparation of the test dispersions in previous studies generally did not include NOM, which could provide nutritional support to *D. magna*, thus reducing their response to carbon nanotubes. The physical properties of MWCNTs also constitute an important factor affecting their ecotoxicity [4].

In addition to the characterization of the dispersions at the end of the ecotoxicity tests, optical microscopy was conducted on daphnids to analyze the presence of attached MWCNTs agglomerates (Figure 5). Dead *Daphnia* were selected for the imaging with the aim of visualizing the differences with live organisms in controls and organic matter solutions.

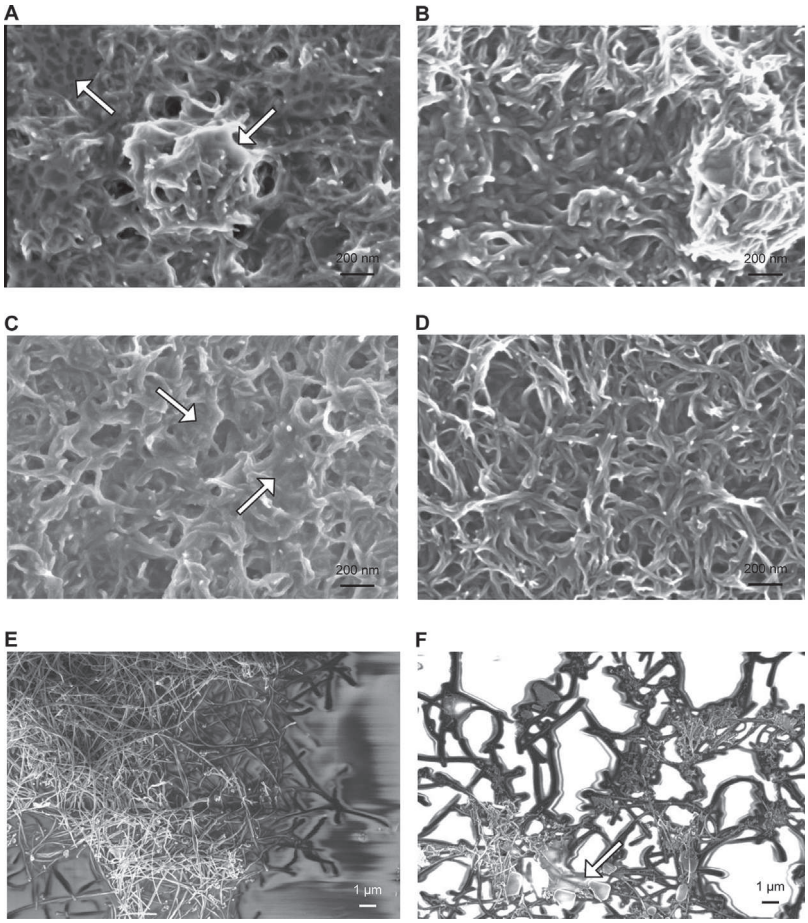

Figure 4. Scanning electron microscope images of the 50 mg/L multiwalled carbon nanotube batch dispersions at end of the tests: (**A**) CNT-1-SR-NOM 48 h, (**B**) CNT-1-HA 48 h, (**C**) CNT-3-SR-NOM 48 h, (**D**) CNT-3-HA 48 h, (**E**) CNT-4-SR-NOM 48 h, (**F**) CNT-4-HA 48h. CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

The dispersions prepared with SR-NOM showed agglomerates of MWCNTs on the body surface (Figure 5C) and antennae of the organisms (red circles in Figure 5B), and the accumulation of nanotubes on their external surface has been observed to be a potential mechanism of toxicity [46]. However, in the case of humic acid dispersions, a greater amount of MWCNTs was observed in the digestive tract of daphnids, given their dark coloration (Figure 5E and 5F). Nonetheless, the organisms exposed to humic acid alone as background substance (Figure 5D) also showed this dark coloration. This fact could pose a greater uptake of humic acid by *D. magna* as a food source and thus explain the reduction in immobilization with respect to MWCNTs dispersed in SR-NOM solutions. The key driving force for the sorption of NOM to MWCNTs is the alkyl (aliphatic) components rather than the aromatic ones of humic acid. The driving forces for the adsorption of SR-NOM onto MWCNTs might be greater than those for humic acid, thus resulting in different modes of action on daphnids. Humic acid, more loosely adsorbed onto nanotubes, might be used as a nutritional support by daphnids during the test, and this fact may delay the digestion of MWCNTs. Therefore, although MWCNTs would be bioavailable for daphnids, toxicity was not observed during the exposure period.

Although SR-NOM has provided a better capability for the stabilization of MWCNTs, and toxicity results on *D. magna* are consistent with those reported in the literature, further research needs to be conducted in this field. Several key aspects, such as the feeding during assays, have been demonstrated to play an essential role in the toxicity mechanisms of carbon nanotubes toward daphnids [8]. In addition, their adverse effects are exerted to several generations of *D. magna* on their survival, reproduction, and growth [32]. These factors might be considered in future studies to ensure the suitability of SR-NOM for the analysis of the ecotoxicity of MWCNTs toward *D. magna* and other organisms at the base of the food chain.

Table 5. Effective concentration values and lower and upper 95% confidence limits of multiwalled carbon nanotube dispersions (mg/L) for *Daphnia magna* neonates during 48 h

Dispersant	Sample	EC20 (95% CL)	EC50 (95% CL)
SR-NOM	CNT-1	4.03 (3.65–4.45)	>50
	CNT-2	2.94 (2.60–3.31)	>50
	CNT-3	ND	ND
	CNT-4	1.08 (0.86–1.35)	27.05 (21.47–34.08)
	SR-NOM	>20	>20
HA	CNT-1	333.15 (315.66–351.60)	>>50
	CNT-2	ND	ND
	CNT-3	ND	ND
	CNT-4	ND	ND
	HA	>20	>20

EC20, EC50 = effective concentrations causing 20% and 50% immobilization, respectively; CL = confidence limit; ND = not determined (effective concentration values could not be calculated because of the low toxicity levels and the scattered points obtained in the dose-response curves); SR-NOM = Suwannee River natural organic matter; CNT = carbon nanotube; HA = humic acid.

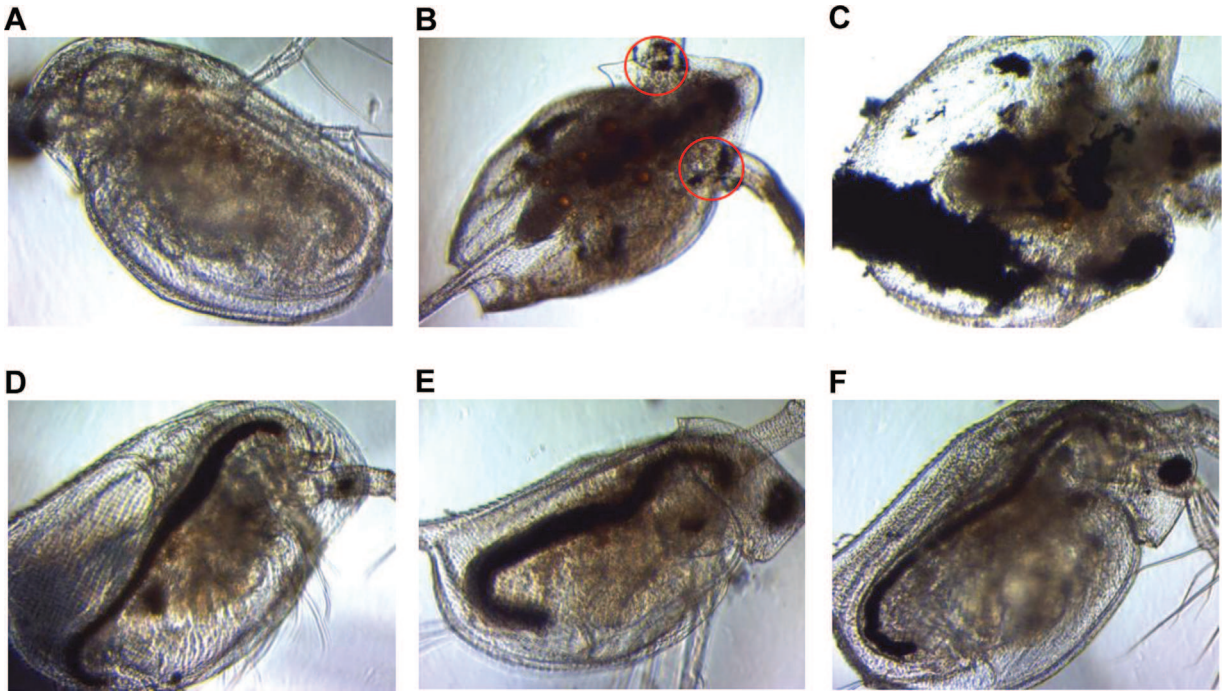

Figure 5. Optical microscope images of *Daphnia magna* exposed to the multiwalled carbon nanotube dispersions at the end of the tests: (**A**) control, (**B**) dead *Daphnia* exposed to 50 mg/L CNT-1 in SR-NOM, (**C**) dead *Daphnia* exposed to 50 mg/L CNT-4 in SR-NOM, (**D**) live *Daphnia* exposed to 20 mg/L HA (background substance), (**E**) dead *Daphnia* exposed to 0.5 mg/L CNT-3 in HA, (**F**) dead *Daphnia* exposed to 5 mg/L CNT-4 in HA. CNT = carbon nanotube; SR-NOM = Suwannee River natural organic matter; HA = humic acid.

CONCLUSIONS

The characterization performed in the present study indicates that NOM provides an increased stability to the MWCNTs' dispersions with respect to synthetic organic matter. Suwannee River-NOM produced a decrease in Z_{ave} or polydispersity index values for all of the nanotubes studied, and also greater and more uniform UV/Vis absorbance results than humic acid. In addition, SEM imaging indicated a higher adsorption of SR-NOM on nanotubes. The outcomes of the toxicity assays confirmed the previously reported finding that increased stability leads to higher inhibitory effects on *D. magna*, because MWCNTs dispersed with SR-NOM exhibit greater toxicity levels than those dispersed with humic acid. The latter seemed to alter the response of the organisms to carbon nanotubes compared with that shown in the presence of SR-NOM. Furthermore, the results obtained with NOM allowed observing the important role of the outer diameter and content of impurities of MWCNTs in their stability and ecotoxicity on daphnids. Suwannee River-NOM is considered to be more appropriate than Sigma-Aldrich humic acid for the ecotoxicity assessment of MWCNTs, not only because of the stability provided to the dispersions, but also because of its capability of simulating the real conditions in aquatic ecosystems.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3172.

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Data availability—Data, associated metadata, and calculation tools are available through the supplemental files, and from the corresponding author (cristina.cerrillo@tekniker.es).

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SUPPLEMENTAL DATA

Colloidal stability and ecotoxicity of multiwalled carbon nanotubes: Influence of select organic matters

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MATERIALS AND METHODS

Materials for dispersions

Table S1. Suppliers of the MWCNTs studied and impurities associated with them

MWCNT code	Supplier identification	Impurities (remaining catalyst metals and amorphous carbon) (%)
CNT-1	Nanocyl NC7000	<5
CNT-2	Arkema Graphistrength C100	<10
CNT-3	JRCNM04000a	16.2
CNT-4	JRCNM04001a	18.1

Preparation of MWCNTs dispersions: Calculation of the total amount of energy delivered by the sonicator probe from calorimetry.

A 600 mL borosilicate glass beaker was filled with 500 mL thermally equilibrated MilliQ water. Its temperature and mass were measured with an uncertainty of ±0.1 ºC and ±0.1 g, respectively. The beaker was placed in the sonicator chamber and the tip was immersed to a position 2.5 cm below the liquid surface. The temperature probe was mounted (using a clamp) at 2.5 cm depth and 1 cm away from the sonicator probe. The sonicator output selected was 40% amplitude (considering previous dispersion tests carried out in our laboratory), operating in continuous mode. The temperature increase of the water was recorded for 6.5 minutes with a time resolution of 30 seconds.

Calculation of the delivered acoustic energy was performed obtaining the best linear fit (R2>0.990) between the measured temperature and time using least squares regression. The effective delivered power was determined using the Equation S1:

— (S1)

where P is the delivered acoustic power (W), dT/dt is the slope of the regression curve, M is the mass of liquid (g), and C_p is the specific heat of the liquid ($J \cdot g^{-1} \cdot ºC^{-1}$).

The effective delivered acoustic power (P) was 25.985 W. The linear fits between the measured temperature as function of time using least squares regression are represented in Figure S1.

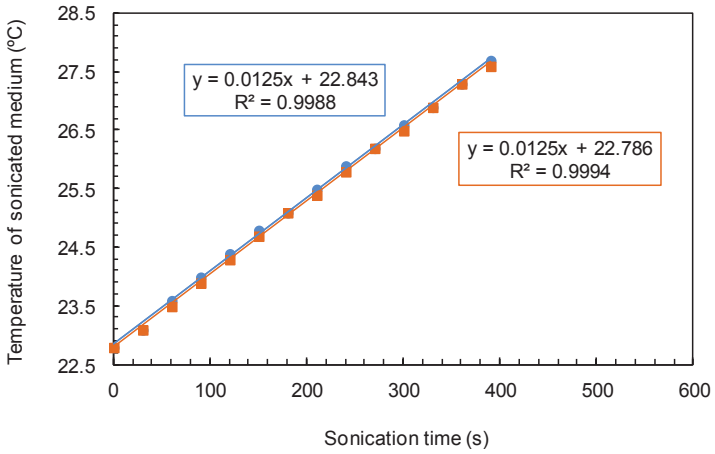


Figure S1. Linear fits between the measured temperature as function of time sonicator probe.

The total amount of energy delivered (Eqn. S2) was obtained considering the applied power and also the total amount of time that the water is subjected to the ultrasonic treatment

(S2)

where E is the total amount of energy (J), P is the delivered acoustic power (W) and t is the total amount of time (s).

Considering the sonication time selected (16 min), the total amount of energy delivered (E) was 24946 J for sonicator probe. These values are in accordance with the maximum specified (30 KJ), to avoid damaging and cutting of CNTs. It is important to consider that the actual volumes and temperatures of MWCNTs dispersions were different from that used in the calculation of the energy delivered by the sonication methods. However, this aspect is noted in the calorimetric method [1], which is simply intended to allow the reporting and transference of sonication power levels between users, not to measure the actual fraction of power utilized for powder disruption under specific dispersion conditions.

Daphnia magna acute immobilization tests

The tubes containing dormant eggs (ephippia) of the organisms were stored in a refrigerator at 5±2 °C. The hatching of the daphnids was carried out by transferring the ephippia into 30 ml culture medium (composition detailed in the next section), 72 h prior to the start of the toxicity tests. They were maintained at 20±2 °C under continuous illumination of a minimum of 80 μE·m-2·s-1 (light intensity at the top of the cultures, measured in the wavelength range of 400-700 nm). The largest hatching occurred between 72 h and 80 h of incubation and the organisms were collected at the latest 90 h after the start of the incubation. Prior to the test, a 2h pre-feeding was applied with a suspension of Spirulina microalgae. This food uptake provided neonates with an energetic reserve and precluded mortality by starvation (which would bias the test results), since the organisms were not fed during the subsequent test. Culture medium was used to prepare HA/SR-NOM solutions for MWCNTs dispersions. Prior to the immobilization tests, the pH of the test dispersions was adjusted to 8.3. The organisms were exposed to five dilutions of the test substances over a period of 48 hours in multiwell test plates. For this purpose, each well was filled with 10 mL of the respective concentrations, in the sequence of increasing toxicant dilutions and five neonates were transferred into each well with a micropipette. The controls and each test concentration were assayed in four replicates (with 5 neonates each well) for a statistically acceptable evaluation of the effects. A Parafilm strip (plastic paraffin film) was put on the plates, and they were covered tightly. Incubation was performed at 20±2 °C in darkness. After 24h and 48h incubation, the multiwell plates were positioned on the stage of a light table and the number of dead and immobilized neonates in each well was recorded. The neonates which were not able to swim after gentle agitation of the liquid for 15 seconds were considered to be immobilized, even if they could still move their antennae.

Preparation of the OECD culture medium for Daphnia magna *(ISO Test water (1); Annex 3 OECD-202).*

The culture and dilution medium for D. magna was prepared by adding 25 mL of the stock solutions 1-4 (they were stored in the dark at 4 °C) to 1 L ultrapure water.

-Stock solution 1: 11.76 g $CaCl_2 \cdot 2H_2O$ in 1 L ultrapure water

-Stock solution 2: 4.93 g $MgSO_4 \cdot 7H_2O$ in 1 L ultrapure water

-Stock solution 3: 2.59 g $NaHCO_3$ in 1 L ultrapure water

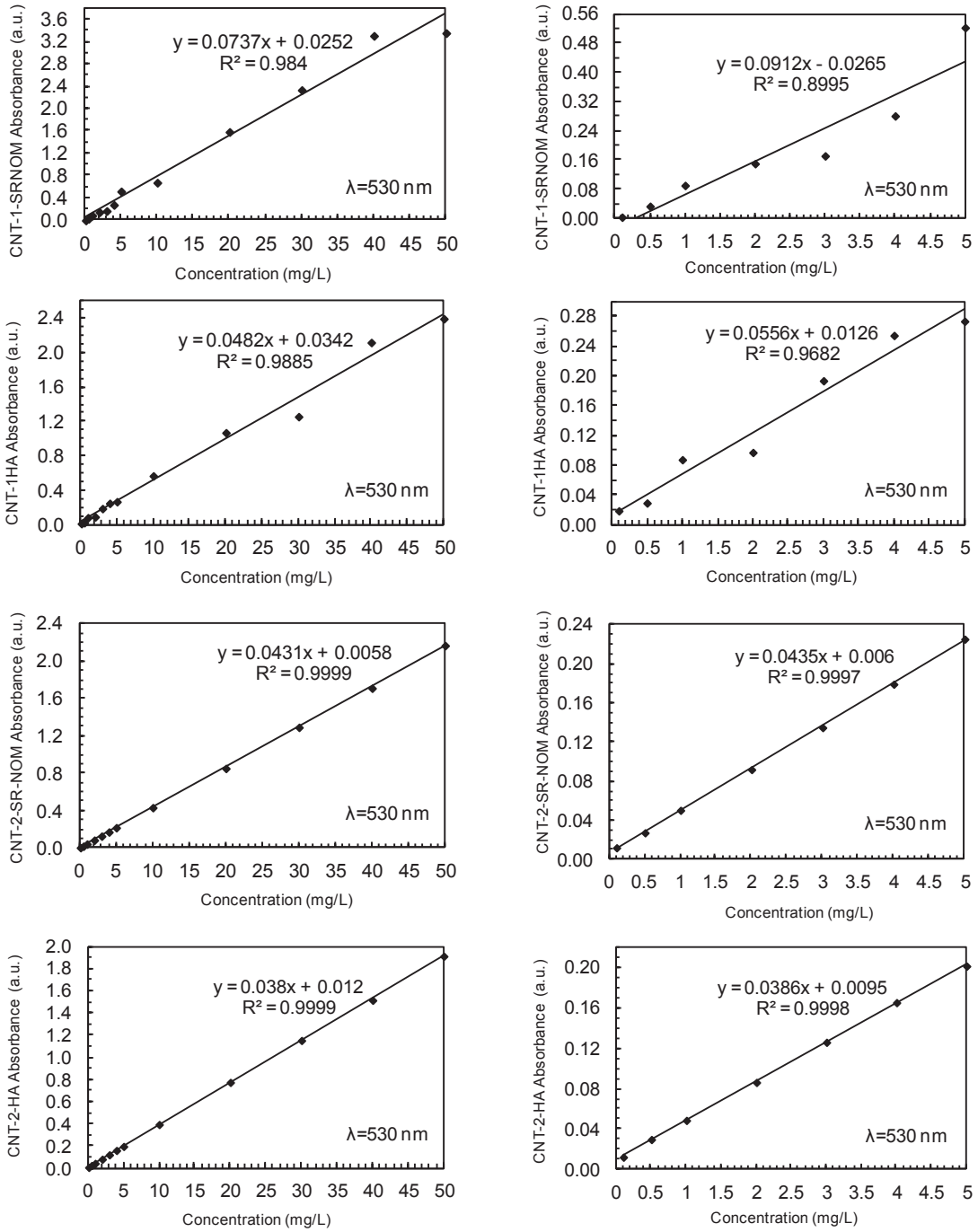
-Stock solution 4: 0.23 g KCl in 1 L ultrapure water

Before use, the solution was equilibrated by bubbling with air for at least 15 minutes. The dissolved oxygen concentration was around 7 mg/L. After equilibration, the pH was adjusted to 8.3, with either 1 M HCl or 1 M NaOH.

RESULTS AND DISCUSSION

Characterization of MWCNTs dispersions

Calibration standards were made by diluting the 50 mg/L MWCNTs dispersions with 20 mg/L HA and SR-NOM solutions prepared in ultrapure water, obtaining eleven levels more: 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5 and 0.1 mg/L. The obtained absorbance values for each concentration are specified in Figure S2.



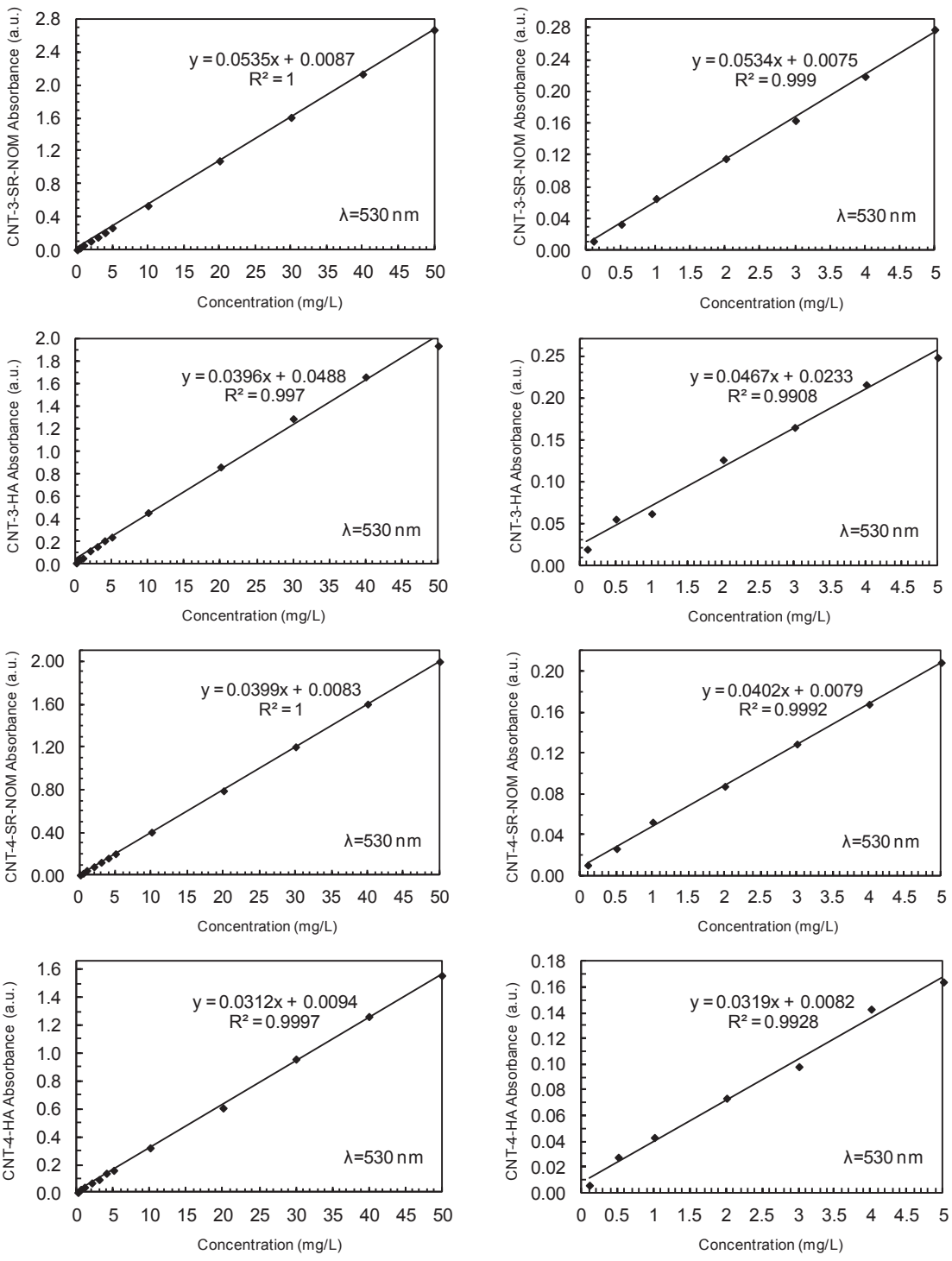


Figure S2. Calibration curves obtained from absorbance of CNTs dispersions with HA and SR-NOM, in different ranges of dilution levels (0.1 to 50 mg/L). The straight lines are linear least-squares fit to the data.

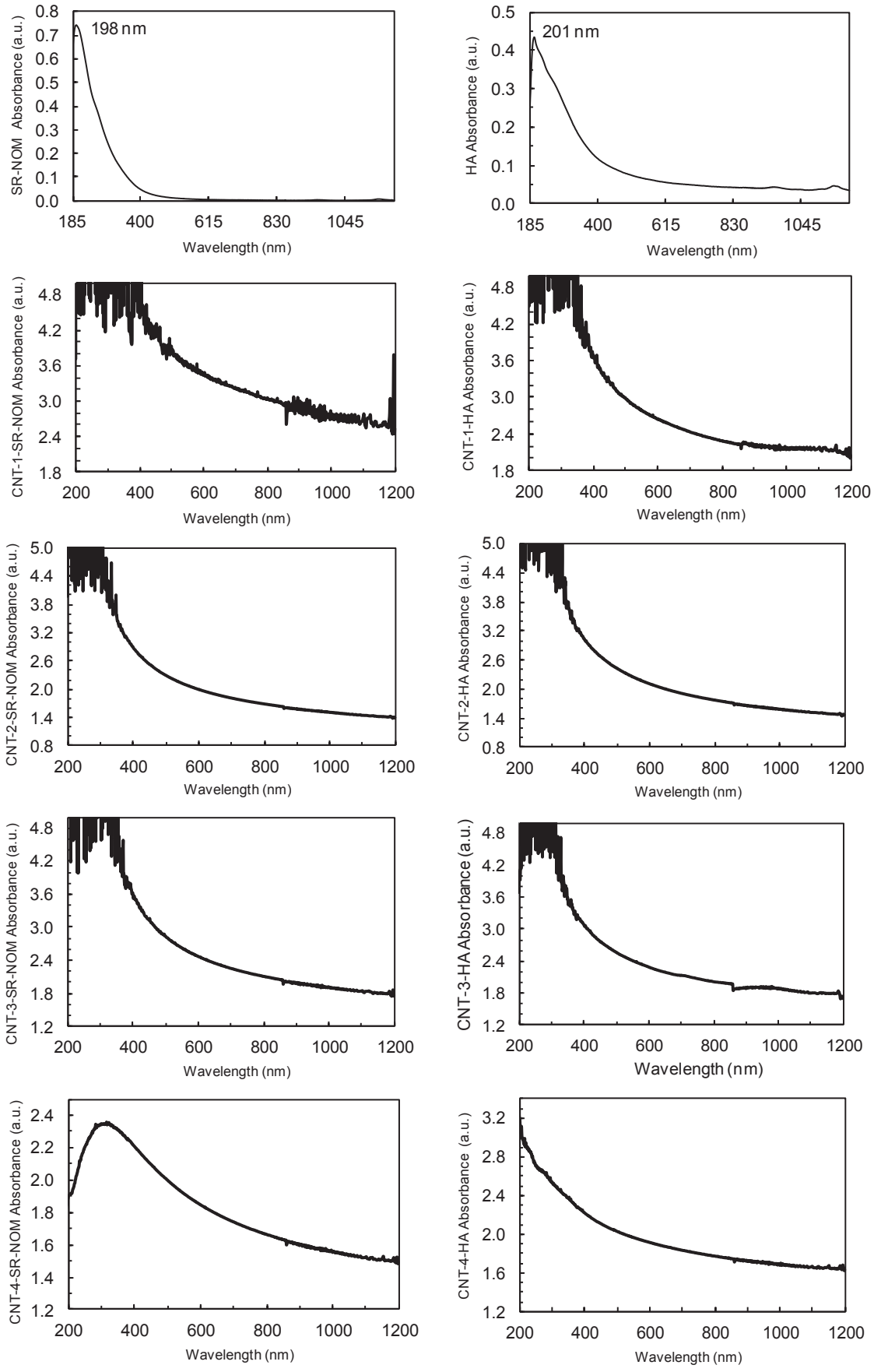
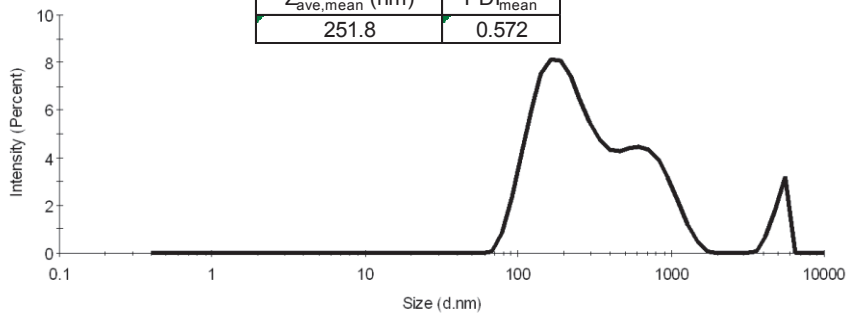
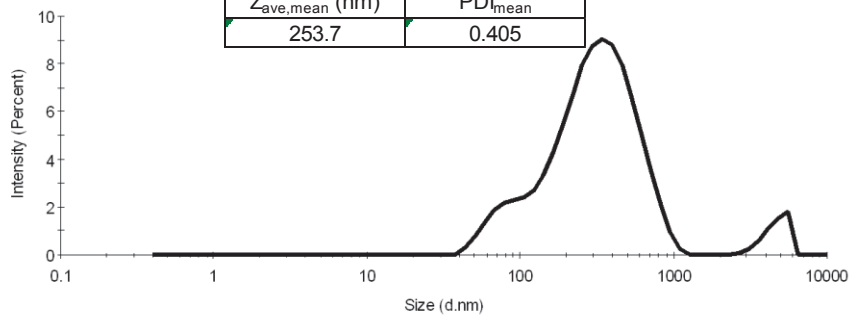


Figure S3. UV/Vis spectra of MWCNTs dispersions considering HA and SR-NOM as background solution.

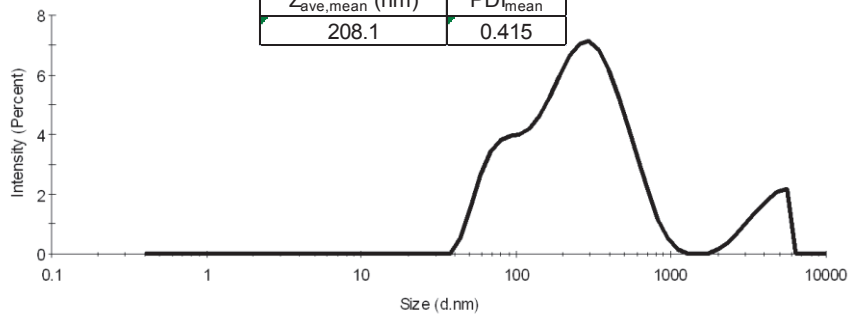
CNT-1-HA_Calibration_50 mg/L	
$Z_{ave,mean}$ (nm)	PDI_{mean}
251.8	0.572



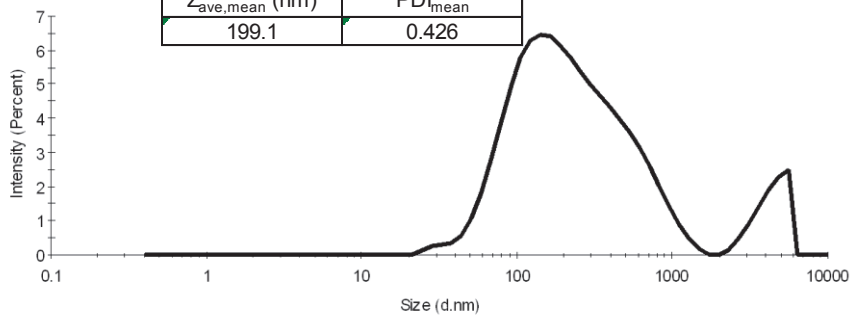
CNT-1-SRNOM_Calibration_50 mg/L	
$Z_{ave,mean}$ (nm)	PDI_{mean}
253.7	0.405



CNT-2-HA_Calibration_50 mg/L	
$Z_{ave,mean}$ (nm)	PDI_{mean}
208.1	0.415



CNT-2-SRNOM_Calibration_50 mg/L	
$Z_{ave,mean}$ (nm)	PDI_{mean}
199.1	0.426



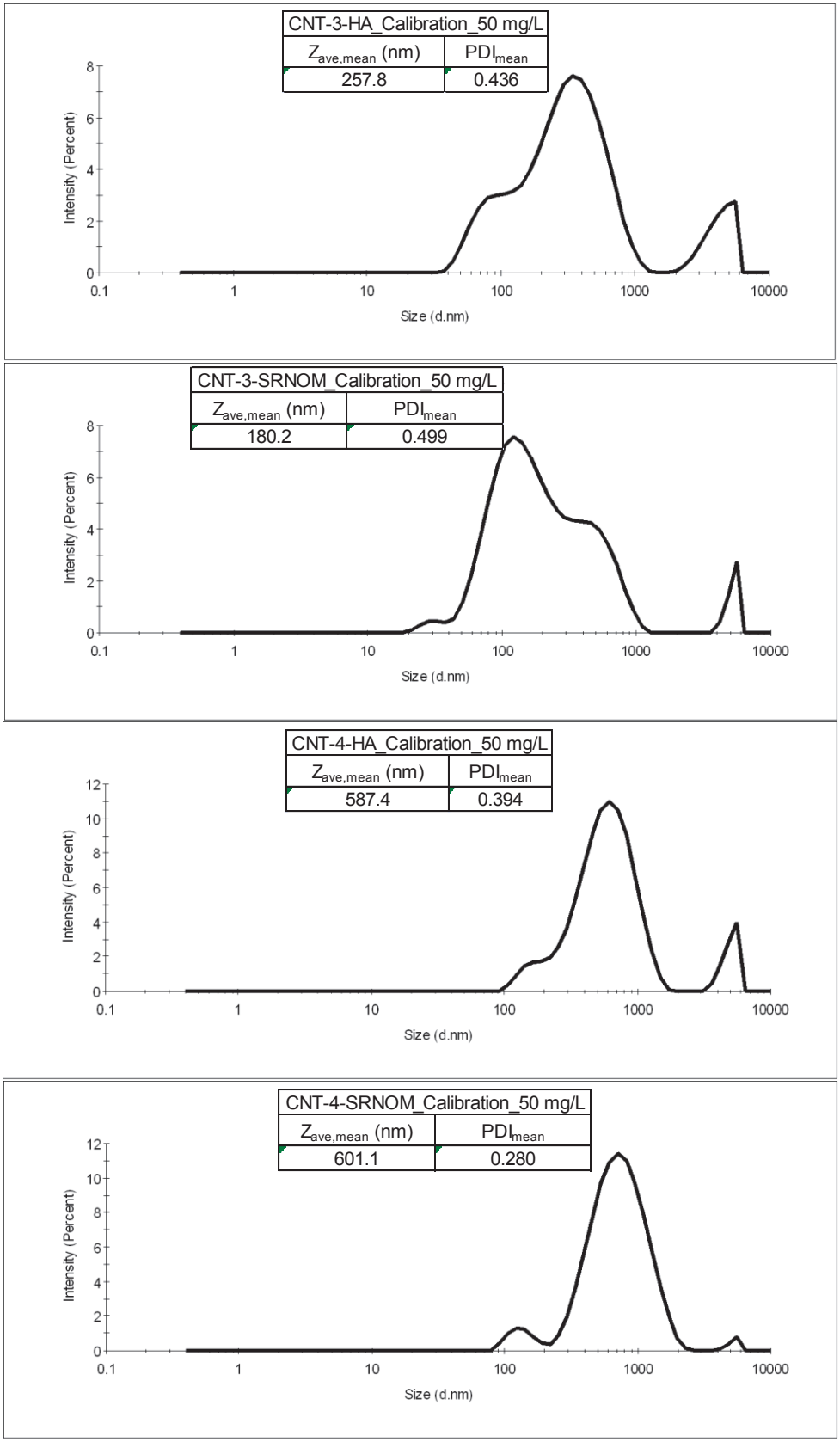


Figure S4. Size distributions by intensity of nanotubes agglomerates in calibration dispersions.

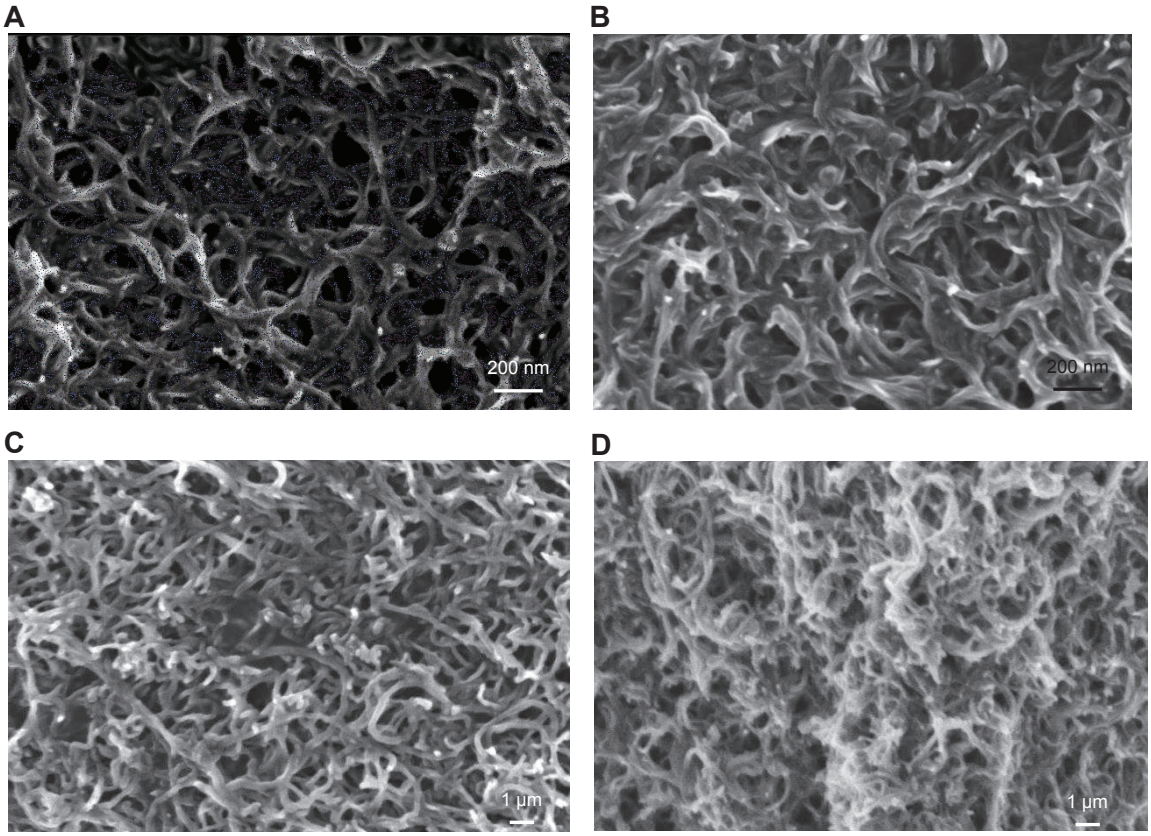


Figure S5. SEM images of the 50 mg/L MWCNTs batch dispersions: (**A**) CNT-3-SR-NOM 0h, (**B**) CNT-3-HA 0h, (**C**) CNT-2-SR-NOM 48h, (**D**) CNT-2-HA 48h.

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Towards the standardization of nanoecotoxicity testing: Natural organic matter 'camouflages' the adverse effects of TiO_2 and CeO_2 nanoparticles on green microalgae

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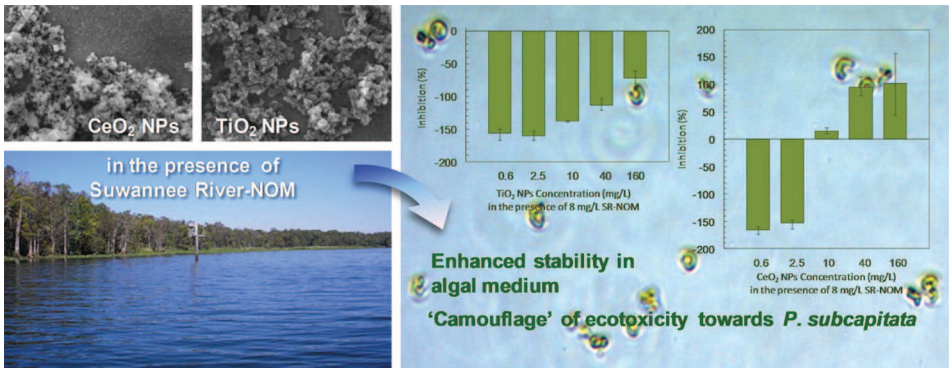
HIGHLIGHTS

- SR-NOM increased significantly the stability of CeO_2 and TiO_2 NPs in algal medium.
- The enhanced stability led to a better reproducibility of the toxicity test results.
- SR-NOM reduced the toxic effect of CeO_2 NPs on algae and eliminated that of TiO_2 NPs.
- This 'camouflage' of toxicity occurred even for low NOM environmental concentrations.
- SR-NOM might be used as a model NOM in standardized ecotoxicity tests of these NPs.

GRAPHICAL ABSTRACT



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ABSTRACT

In the last few years, the emission of CeO_2 and TiO_2 nanoparticles (NPs) into the environment has been raising concerns about their potential adverse effects on wildlife and human health. Aquatic organisms constitute one of the most important pathways for the entrance of these NPs and transfer throughout the food web, but divergences exist in the experimental data published on their aquatic toxicity. The pressing need for standardization of methods to analyze their ecotoxicity requires aquatic media representing realistic environmental conditions. The present study aimed to determine the usefulness of Suwannee River natural organic matter (SR-NOM) in the assessment of the agglomeration kinetics and ecotoxicity of CeO_2 and TiO_2 NPs towards green microalgae *Pseudokirchneriella subcapitata*. SR-NOM alleviated the adverse effects of NPs on algal growth, completely in the case of TiO_2 NPs and partially in the case of CeO_2 NPs, suggesting a 'camouflage' of toxicity. This behavior has been observed also for other algal species and types of natural organic matter in the literature. Furthermore, SR-NOM markedly increased the stability of the NPs in algal medium, which led to a better reproducibility of the toxicity test results, and provided an electrophoretic mobility similar to that previously reported in various river and groundwaters. Thus, SR-NOM can be a representative sample of what is found in many different ecosystems, and the observed 'camouflage' of the effects of CeO_2 and TiO_2 NPs on algal cells might be considered as a natural interaction occurring in their standardized ecotoxicological assessment.

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1. Introduction

Nanoscale CeO_2 and TiO_2 are two of the most extensively manufactured nanomaterials (MNMs) used currently. They are incorporated into a wide variety of products, including catalysts, gas sensors, solar cells, oxygen pumps, fuels in the automotive industry, paints, coatings and cosmetics (Klaine et al., 2008; Yadav and Mungray, 2014). Consequently, the constant increase in their large scale production and their inherent emission into the environment are raising concerns regarding their potential adverse effects on wildlife and human health (Keller et al., 2010; Keller et al., 2013).

Aquatic organisms constitute one of the most important pathways for the entrance and transfer of MNMs throughout the food webs in ecosystems (Baun et al., 2008). The data published on the aquatic toxicity of CeO_2 and TiO_2 nanoparticles (NPs) are divergent (Menard et al., 2011; Booth et al., 2015) and this limited understanding of their impacts poses a barrier to their current and potential applications. Factors such as physico-chemical properties (size, shape and surface chemistry) and environmental conditions (pH, ionic strength, colloids and natural organic matter concentration) play an important role on the fate and toxic effects of these NPs (Keller et al., 2010; Baun et al., 2008; Menard et al., 2011). The combination of these properties and conditions may result in either their agglomeration or stabilization, affecting their bioavailability and determining their toxicity. If the degree of agglomeration of the NPs in the test media is not representative of that occurring in natural waters, the current regulatory testing can under- or overestimate their toxicity to aquatic organisms, which is considered as a prominent concern within the scientific community (Park et al., 2014). Therefore, the pressing need for standardization of methods to analyze the ecotoxicity of CeO_2 and TiO_2 NPs (Savolainen et al., 2013; Van Hoecke et al., 2011) requires media which better represent the behavior of MNMs in realistic environmental conditions.

The interaction of natural organic matter (NOM) and its predominant substance, humic acid (HA) (Tang et al., 2014), with MNMs, is an issue extensively analyzed in the literature. NOM may influence the stability and toxicity of MNMs and can also play an important role in removing toxic substances from effluents, but further research is needed to better understand these dynamic interactions (Grillo et al., 2015). In the case of CeO_2 and TiO_2 NPs, it has been widely demonstrated that different types of NOM promotes their stabilization in aqueous media at typical environmental concentrations (Keller et al., 2010; Yang et al., 2009; Quik et al., 2010; Erhayem and Sohn, 2014a) and affect also their toxic effects in aquatic and soil organisms (Van Hoecke et al., 2011; Schwabe et al., 2013; Collin et al., 2014a). Likewise, synthetic organic matters have been proved to interact with these NPs, but the influence on their stability and ecotoxicity is not as clear as that of NOM (Schwabe et al., 2013; Zhu et al., 2014; Wang et al., 2014). Currently, several types of NOM are commercially available and their key advantage over laboratory-synthesized substances is a more realistic simulation of the ecosystems in the toxicity assays.

NOM and HA from Suwannee River (IHSS, n.d.) are the most analyzed organic matters in the study of bioavailability of CeO_2 and TiO_2 NPs (Booth et al., 2015; Li and Chen, 2012; Quik et al., 2012; Thio et al., 2011; Chowdhury et al., 2012; Loosli et al., 2013; Yang et al., 2013; Cupi et al., 2015; Dasari and Hwang, 2013; Neale et al., 2015; Mwaanga et al., 2014). A recent research on nano-TiO_2 stability upon adsorption of Suwannee River humic substances concluded that Suwannee River NOM (SR-NOM) was the most representative sample of what is found naturally and would likely provide the most useful outcomes (Erhayem and Sohn, 2014a). Hence, SR-NOM might fulfill the need for standardization mentioned above. Nonetheless, further research is still required to prove its suitability in the ecotoxicological assessment of MNMs. As reported by us elsewhere (Cerrillo et al., 2015a), SR-NOM provides an increased colloidal stability to multiwalled carbon nanotubes with respect to synthetic HA, which resulted in higher adverse effects on *Daphnia magna*. However, Cupi et al. (Cupi et al., 2015) observed that the addition of SR-NOM alleviated Ag NPs toxicity towards *Daphnia magna*, and caused agglomeration and settling of TiO_2 NPs in their culture medium. They highlighted the lack of studies that systematically investigate the stability of NP dispersions in the presence of NOM and its implications in the toxicity tests outcome, and suggested that SR-NOM should be added only in certain cases. This approach for the standardization of toxicity testing on a case-by-case basis for every possible exposure scenario has been supported also in other studies (Dasari and Hwang, 2013). Although alternative testing strategies have proposed a more efficient assessment of the risks of MNMs (Stone et al., 2014), the current lack of specific tools to identify and predict them makes necessary a comprehensive ecotoxicological assessment of the growing number of MNMs so far, at least for various trophic levels.

Considering that the selection of reference materials and methods to assess the ecotoxicity of CeO_2 and TiO_2 NPs still remains unsolved, the present study aims to serve as a next step towards the establishment of standardized ecotoxicity tests of these nanomaterials. The agglomeration kinetics and ecotoxicity of CeO_2 and TiO_2 NPs towards *Pseudokirchneriella subcapitata* were analyzed in the presence and absence of SR-NOM. These unicellular green algae were selected considering their key role in the aquatic ecosystems and in regulatory testing. The standardization approach of this work also included the calculation of the dispersion parameters required to optimize the energy delivered to the NPs during the preparation of the dispersions.

2. Materials and methods

2.1. Chemicals and materials

CeO_2 and TiO_2 nanoparticles were acquired in powdered form from JRC (Joint Research Centre-European Commission) Repository. Their primary characterization data (Table 1) were also provided by JRC.

Standard Suwannee River NOM (SR-NOM) obtained from the International Humic Substances Society (IHSS) (IHSS, n.d.) was used as a model NOM without any further purification.

All stock dispersions and solutions were prepared in ultrapure water, produced by a Milli-Q water filtration system (Millipore). The rest of chemicals used were p.a. grade and obtained from Sigma-Aldrich and Scharlab.

2.2. Preparation of NP dispersions

The stock dispersions were obtained by adding 10 mL of Milli-Q water to 25.6 mg CeO_2/TiO_2 NPs in 20 mL glass scintillation vials and then sonicating with an ultrasonic homogenizer, a widely accepted method that ensures reasonable stability (Keller et al., 2010; Cupi et al., 2015). It has shown to provide better optimization of the energy delivered to the MNMs than other devices, such as ultrasonic baths (Cerrillo et al., 2015b). The sonicator (VIBRACELL-VCX750, SONICS&MATERIALS) operated with a standard probe (136 mm length and 13 mm diameter), at a frequency of 20 kHz, continuous mode for 12 min and output power fixed at 750 W at 20% amplitude. The calorimetric method described by Taurozzi et al. (2011) was used to calculate the sonication time and amplitude required to obtain agglomerate sizes as near as possible to the nanometric range. Additional details on these calculations are provided in the Supplementary Data and Fig. S1. During

Table 1
Physical descriptions of the NPs studied.

Nanomaterial	Supplier identification	Size (nm)[a]	Surface area (m^2/g)[b]	Impurities (wt.%)
CeO_2	JRCNM02102a	33–49	28	<0.1%
TiO_2	JRCNM01003a	22–27	51	4.1%

[a] Transmission electron microscope (TEM) data on the primary particle size.
[b] Obtained by Brunauer–Emmett–Teller (BET) analysis.

sonication, the vials were held in an ice bath to minimize rising of the temperature of the sample, and the probe was inserted between the upper quarter and upper half of the dispersion volume. These conditions maximized the liquid-probe surface area exposed to the acoustic waves, and the vial wall surface/volume ratio for dissipation of heat by the cooling bath (Taurozzi et al., 2011). Both CeO_2 and TiO_2 NP dispersions were prepared in the same manner and delivered with the same concentration in order to ensure the consistency of test procedures. The high initial concentrations (2560 mg/L) were selected to perform subsequent dilution into the algae growth medium for conducting the ecotoxicity tests, according to the Technical Guidance Document developed in the EU FP7 NANoREG Project (Jensen, 2014). The characterization and ecotoxicological assessment of the NP dispersions was conducted immediately after their preparation.

2.3. Algae ecotoxicity studies

The ecotoxicity of CeO_2 and TiO_2 NPs towards unicellular green algae *P. subcapitata* was determined in the presence and absence of SR-NOM, according to the OECD Guideline 201 "Algal growth inhibition test" (Freshwater Alga and Cyanobacteria, Growth Inhibition Test, 2011). The algal cells were obtained from the CCAP (Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, UK). The tests were conducted in the form of range-finding pre-tests to observe the effects of SR-NOM on a wide range of NP concentrations, since obtaining accurate effective concentration data was not within the aim of the present study. The system response was evaluated as a function of growth of algal cultures exposed to NPs in comparison with the average growth of unexposed control cultures. Additional information on the culturing conditions and preparation of the growth medium are detailed in the Supplementary Data (Table S1). Growth inhibition was quantified at 24, 48 and 72 h, and tentative test endpoints were determined by calculating the concentrations bringing 10% and 50% inhibition (EC10 and EC50, respectively) as well as their associated 95% confidence limits (CL) after the 72 h exposure. These data were determined by regression analysis in Excel 2007 (Microsoft Corporation). Chlorophyll-a extractions were performed to estimate the biomass concentrations of the algal cultures by means of a modified version of the fluorescence method specified in OECD-201 (Mayer et al., 1997). This technique has been previously demonstrated to be useful in the assessment of the effects of CeO_2 and TiO_2 NPs on green algae (Booth et al., 2015; Cardinale et al., 2012). Additional details on the calculations to derive the algal biomass from extracted chlorophyll are provided in the Supplementary Data (Fig. S2).

The test dispersions were prepared by transferring the required volume of stock dispersions of NPs into 250 mL Erlenmeyer flasks and adding algae growth medium up to the 100 mL mark. The flasks were capped with air-permeable cellulose stoppers to prevent cross-contamination. Exposures were conducted at nominal NP concentrations of 160, 40, 10, 2.5 and 0.6 mg/L. The first level of the dilution series (160 mg/L) was selected according to the short-term endpoints reported in the literature for *P. subcapitata* and CeO_2 and TiO_2 NPs (Menard et al., 2011; Booth et al., 2015; Neale et al., 2015; Collin et al., 2014b). These concentrations exceeded those expected in the environment, but allowed a subsequent comparison of the toxicity results obtained in the present study with previous publications. The test design included three replicates at each test concentration and six control replicates. Two test sets were conducted in the presence of natural organic matter by adding 8 and 20 mg/L of SR-NOM to the growth medium of the organisms previous to the dilution of NP stock dispersions. These concentrations were representative for surface waters (Quik et al., 2010; Cupi et al., 2015), the *P. subcapitata* environments. The amounts of organic carbon in natural surface waters range from 0.5 mg C/L (sea water) to 33 mg C/L (bogs) (Thurman, 1985), which correspond to approximately 1 to 63 mg/L SR-NOM. Independent toxicity tests were performed with Suwannee River NOM to determine its influence in the effects of the NPs studied towards algae.

2.4. Characterization of NP dispersions

Dynamic light scattering (DLS), UV/Vis spectroscopy, and scanning electron microscopy (SEM) characterization were conducted in the NP dispersions immediately after their preparation. The dispersions with the highest exposure concentrations (160 mg/L) were characterized at the beginning and end of the tests to assess their colloidal stability and agglomeration rate as a function of time. The characterization of the dispersions at the end of the tests was preceded by a slight shaking for homogenization. Sampling only the aquatic phase would have required additional settling or centrifugation steps because sedimented or agglomerated MWCNTs were not clearly observed.

The stability of the stock dispersions and the 160 mg/L NP test dispersions was assessed by measuring the variation in calculated average zeta-sizes during the exposure period. For this purpose, Zeta-average diameter (Z_{ave}) and polydispersity index (PDI) were obtained by DLS measurements in a Malvern Zetasizer Nano ZS instrument, considering the data generated from ten repeated measurements. In addition, zeta potentials of the test dispersions were obtained at the beginning of the tests to determine the influence of NOM in the electrophoretic mobility (EPM) of the NPs.

The DLS characterization was supported by qualitative analysis of the agglomeration during the tests, conducted in the 160 mg/L NP dispersions by measuring their total absorbance of light. UV/Vis spectroscopy is a widely used technique to analyze the stability of CeO_2 and TiO_2 NPs, given its rapidness and low cost. An UV/Vis/NIR spectrophotometer (Lambda 950, PerkinElmer) and quartz cells with 10 mm path length were used for this purpose. Calibration curves based on multi-concentration dispersions of CeO_2 and TiO_2 NPs in Milli-Q water (Supplementary Data, Fig. S3) were used as a reference to perform this analysis. The selection of the wavelength was carried out considering the previously reported values for these NPs (Keller et al., 2010; Li et al., 2011; Erhayem and Sohn, 2014b), and the fact that saturation of the spectrophotometer was reached in the regions of the spectrum near their absorbance peaks (approximately 305 nm for both CeO_2 and TiO_2, Supplementary Data, Fig. S4). In addition, the absorbance of organic matter (Supplementary Data, Fig. S5) could have interfered with those of CeO_2 and TiO_2. The absorbance measurements were performed at 400 nm, where the absorbance of SR-NOM was negligible and did not alter those obtained for the NPs studied. Nonetheless, the measurements were carried out taking SR-NOM as background substance and subtracting their absorbance by the "autozero" function of the spectrophotometer. The almost negligible absorbance of the nutrients in the growth medium was also subtracted.

Furthermore, the results obtained in the previous characterization were complemented by SEM imaging, using a ZEISS apparatus (ULTRA PLUS model). The SEM samples were prepared by a drying process for 24 h under ambient temperature.

3. Results and discussion

3.1. Characterization by DLS

The results of the DLS measurements performed in the stock dispersions and the 160 mg/L NP test dispersions are shown in Tables 2 and 3. Size distribution and zeta potential graphs are provided in the Supplementary Data (Figs. S6 to S15). The 8 and 20 mg/L SR-NOM dispersions were labeled as SRNOM-8 and SRNOM-20, respectively.

The agglomerate sizes in stock dispersions were consistent with their nominal particle sizes in the primary characterization, and for both CeO_2 and TiO_2, Z_{ave} was approximately six times greater than the primary NP size (Tables 1 and 2). It was also experimentally observed that the dilution of the stock dispersions into the algal growth medium substantially increased the Z_{ave} of CeO_2 and TiO_2 NPs to a 2–3 μm range. This behavior, caused by the nutrients present in the medium, was significantly altered in the presence of both 8 mg/L and 20 mg/L SR-NOM,

Table 2
Zeta-average diameters (Z_{ave}) and polydispersity indices (PDI) of the stock dispersions and 160 mg/L NP test dispersions at the beginning and end of the tests.

	CeO_2 NPs				TiO_2 NPs			
	$Z_{ave,mean}$ (nm)	SD	PDI_{mean}	SD	$Z_{ave,mean}$ (nm)	SD	PDI_{mean}	SD
Stock dispersions	196.8	9.6	0.345	0.056	151.6	1.0	0.334	0.008
Test dispersions—0 h	2557.7	613.8	0.301	0.040	2389.3	376.4	0.244	0.040
Test dispersions—72 h	1939.7	307.7	0.279	0.017	2697.0	368.6	0.203	0.042
Test dispersions + SRNOM-8-0 h	176.4	4.5	0.234	0.007	175.3	8.9	0.259	0.056
Test dispersions + SRNOM-8-72 h	235.3	17.1	0.219	0.015	178.7	10.9	0.270	0.023
Test dispersions + SRNOM-20-0 h	167.9	7.1	0.215	0.025	152.6	1.9	0.245	0.007
Test dispersions + SRNOM-20-72 h	259.4	8.7	0.235	0.012	173.8	29.2	0.316	0.056

SD = standard deviation of measurements corresponding to three test replicates.

since the NPs maintained approximately the same agglomerate sizes obtained in stock dispersions even at the end of the exposure period (Fig. 1A and B). This outcome demonstrated the increased stability provided by SR-NOM to CeO_2 and TiO_2 dispersions, which can be explained on the basis of the strong adsorption of organic matter to metal oxide nanoparticles (Quik et al., 2010; Erhayem and Sohn, 2014a). Increasing SR-NOM concentrations resulted in a slight decrease of Z_{ave} in most cases, but these variations were negligible in the range of the agglomerate sizes obtained.

Concerning the PDI, the low values obtained for the stock dispersions and over the duration of the tests (between 0.203 and 0.345) indicated that DLS was a suitable technique to determine the stability of the NPs in this study. High PDI is considered a limiting factor for the use of DLS in particle size characterization (Klaine et al., 2008; Cerrillo et al., 2015a). The test dispersions showed lower polydispersity than the stock dispersions in all cases (Figs. 1C and D). These narrower size distributions might be a result of dilution itself from 2560 mg/L to 160 mg/L, although the presence of the nutrient salts in the algae growth medium could also influence polydispersity. The presence of nutrient salts, which determine the ionic strength of the aqueous medium, and the MNM concentrations have been reported to affect their agglomeration kinetics and stability (Keller et al., 2010; Van Hoecke et al., 2011; Zhu et al., 2014; Mwaanga et al., 2014). Nonetheless, the PDI values in the presence and absence of SR-NOM were different for CeO_2 and TiO_2 NPs. A decreasing trend was observed in the polydispersity of the CeO_2 test dispersions after adding SR-NOM, whilst TiO_2 showed the opposite behavior. This fact might constitute an indicator of the different effects of SR-NOM on the agglomeration kinetics and ecotoxicity of different NPs, mentioned in Section 1. The higher values observed for PDI of TiO_2 dispersions in the presence of SR-NOM were probably caused by the exopolymeric substances excreted by algae to mitigate the stress induced, cited as a contributor to agglomeration (Hartmann et al., 2010). These exudates are considered a much bigger problem for nanomaterials experiments compared to traditional chemicals (Handy et al., 2012), and might be more abundant in TiO_2 NP dispersions, considering the high algal growth rates in the presence of SR-NOM (see Section 3.4). This behavior can also be related to the critical coagulation concentration of TiO_2 NPs, which have shown higher sedimentation rates than CeO_2 NPs in natural aqueous media (Keller et al., 2010).

Regarding the variation in calculated DLS parameters as a function of time, a slight increase in the agglomerate sizes and PDI after 72 h of exposure was observed in the presence of SR-NOM in most cases. It was probably because the alterations in the algal growth introduced by the organic matter (see Section 3.4) or the above mentioned presence of exudates might contribute to agglomeration. In the tests performed in the absence of organic matter, PDI showed the opposite trend and decreased at the end of the exposure period. Z_{ave} did not show a clear trend over the test duration and presented considerable standard deviation. This fact indicated that SR-NOM not only improved the stability of the dispersions, but also contributed to a better homogeneity of the DLS results over time.

The DLS results obtained in the present study were similar to those previously reported in the literature. Zeta-average diameters and PDI of CeO_2 and TiO_2 NPs were influenced mainly by their physicochemical properties, the methods and growth medium used to prepare the dispersions, and the type of NOM added. CeO_2 NPs, with primary particle size of 20 nm studied by Quik et al. (2010), reduced their Z_{ave} in *P. subcapitata* growth medium from 417 nm to 248 nm after adding SR-NOM. Cupi et al. (2015) also analyzed the SR-NOM effect on the agglomerate sizes of 25-nm-diameter TiO_2 NPs, and observed a decrease in Z_{ave} from 1325 nm to 101 nm (maximum and minimum values obtained, respectively) in *D. magna* medium. Nevertheless, these studies did not provide the amount of acoustic energy delivered to the NPs during the preparation of dispersions, which determines the hydrodynamic particle size-distributions. The sonicator's power setting value does not indicate itself accurately the effective acoustic power (Taurozzi et al., 2011). The standardization approach of the present study included the calculation of the energy delivered to the NPs during the preparation of dispersions (see Section 2) to allow a fully reproducible method. With respect to PDI, Cupi et al. (2015) obtained values of up to 0.88 and 0.39 in the absence and presence of SR-NOM, respectively (which were not as low as the values observed in the present study), and Quik et al. (2010) did not provide them. This fact suggests a better optimization of the energy delivered to the NPs in the dispersion process.

The electrophoretic mobility and the zeta potential values obtained (Table 3 and Fig. 1E) showed that CeO_2 NPs exhibited higher stability than TiO_2 NPs in algal growth medium alone. The reason underlying might be the high tendency of CeO_2 NPs to adsorb phosphate ions dissolved in the algal growth medium (Booth et al., 2015; Van Hoecke et al., 2009). The addition of SR-NOM resulted in even more negative zeta potentials for both CeO_2 and TiO_2 test dispersions. SR-NOM concentration did not influence EPM significantly, considering the negligible

Table 3
Zeta potential and electrophoretic mobility of the 160 mg/L NP test dispersions at the beginning of the tests.

	CeO_2 NPs				TiO_2 NPs			
	Zeta potential ζ (mV)	SD	EPM U (μm cm V^{-1} s^{-1})	SD	Zeta potential ζ (mV)	SD	EPM U (μm cm V^{-1} s^{-1})	SD
Test dispersions	−19.9	0.2	−1.562	0.015	−3.3	0.4	−0.257	0.033
Test dispersions + SRNOM-8	−29.7	0.7	−2.329	0.051	−25.8	0.4	−2.025	0.030
Test dispersions + SRNOM-20	−28.5	0.3	−2.233	0.025	−24.6	0.8	−1.926	0.063

SD = standard deviation of measurements corresponding to three test replicates.
EPM = Electrophoretic mobility.

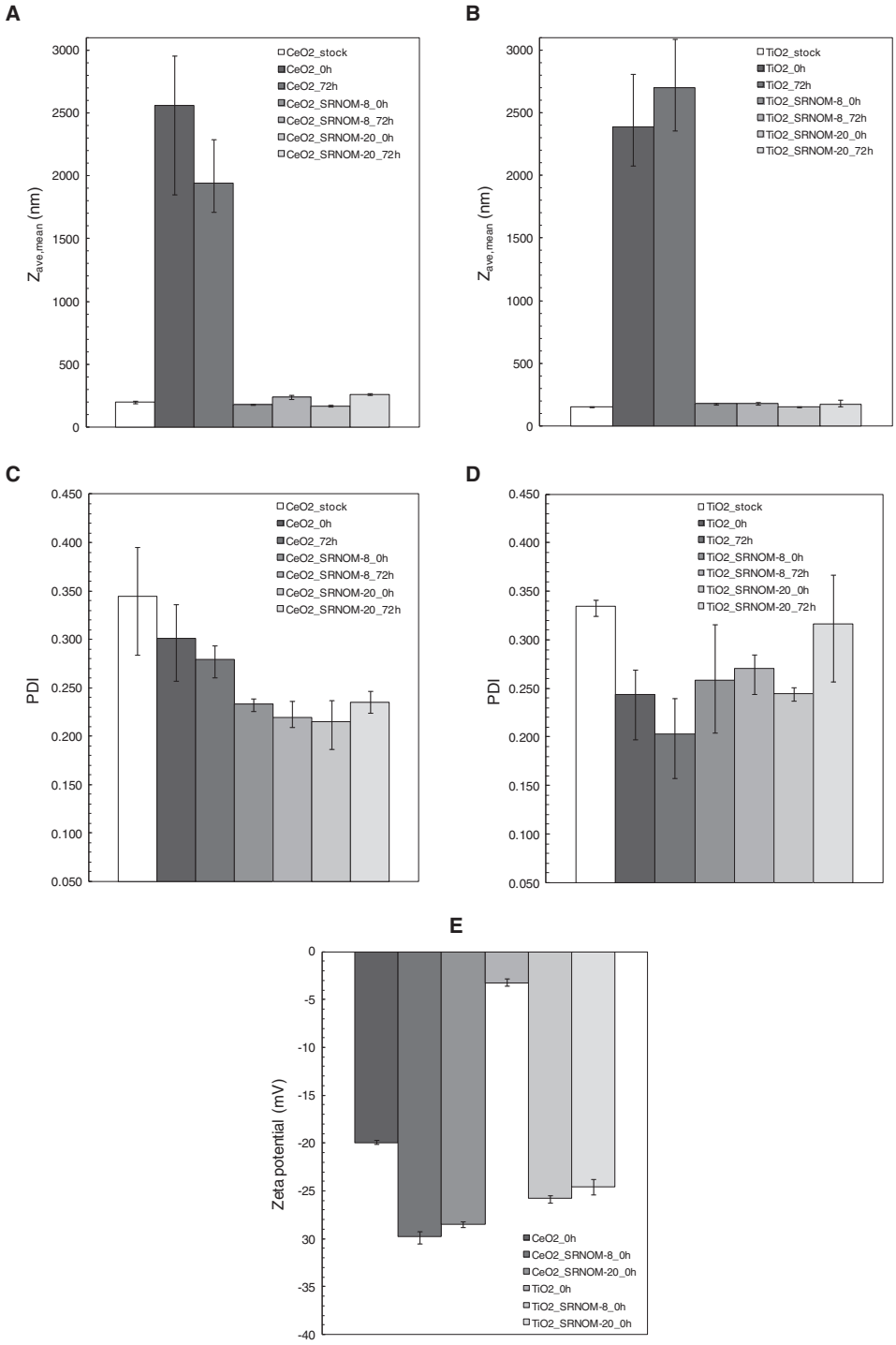

Fig. 1. Histogram comparisons of Z_{ave} size (A,B), PDI (C,D) and zeta potential (E) of the stock dispersions and 160 mg/L NP test dispersions at the beginning and end of the tests. Error bars represent standard deviation (n = 3).

differences and the standard deviations observed in zeta potentials of 8 mg/L and 20 mg/L SR-NOM samples. The dramatic enhancement of the TiO_2 NPs EPM after adding SR-NOM was consistent with the more pronounced reduction in its Z_{ave} compared to that of CeO_2 NPs (see Figs. 1A and B). This behavior could be related to the greater particle surface area of TiO_2 NPs, which might lead to an increase of the amount of SR-NOM adsorbed with respect to CeO_2 NPs (see Table 1). Additional SEM characterization was conducted on stock dispersions of NPs to further analyze this phenomenon at the nanoparticle level (Fig. S16). The considerable variability observed in CeO_2 primary particle sizes influenced their low particle surface area compared to that of TiO_2 NPs. Furthermore, the round or elongated shape of TiO_2 NPs probably promoted the adsorption of SR-NOM, whilst the polyhedral morphology of CeO_2 NPs hindered their interaction with SR-NOM because of a directional adsorption mechanism. Electrophoretic mobility of CeO_2 and TiO_2 NP dispersions tended to similar values in the presence of SR-NOM. A previous study on this issue (Keller et al., 2010) reported the same behavior for EPM of CeO_2 and TiO_2 NPs in various river and groundwaters. This fact suggests that SR-NOM might be a representative sample of what is found in many different ecosystems, and hence fulfill the need

for standardization of ecotoxicity tests. The zeta potential values reported in the literature for CeO_2 NPs in the presence of SR-NOM (Quik et al., 2010), and for TiO_2 NPs in the presence of humic acids from Suwannee River (Thio et al., 2011; Loosli et al., 2013) were also in accord with the ranges observed in the present study.

3.2. Characterization by UV/Vis spectroscopy

Generally, the UV/Vis characterization supported the results obtained with DLS measurements. The more pronounced changes in the electrophoretic mobility and Z_{ave} of TiO_2 NPs compared to that of CeO_2 NPs in the presence of SR-NOM were in good agreement with their greater absorbance after adding organic matter (see Fig. 2).

The fact that low Z_{ave} results in higher UV/Vis absorbances is generally accepted. Large agglomerates are more prone to destabilization and sedimentation, which result in lower UV/Vis absorbance (Keller et al., 2010; Cerrillo et al., 2015a; Erhayem and Sohn, 2014b). The increase observed in the absorbance values of TiO_2 dispersions might be caused by their smaller agglomerate sizes with respect to those of CeO_2 dispersions, apart from their crystalline structure, which also determines their UV/Vis spectra (Supplementary Data, Fig. S4). In the case of CeO_2, the test dispersions prepared in the presence of SR-NOM showed lower optical absorbance with respect to those prepared in growth medium alone. The considerable standard deviation obtained in the absence of SR-NOM constitutes an indicator of the instability of these dispersions and might explain these anomalous results. Their limited reliability was also supported by the similar absorbance of CeO_2 test dispersions in the presence of organic matter and that of 160 mg/L calibration standards. SR-NOM concentrations did not appear to substantially impact the absorbance values of CeO_2 and TiO_2 dispersions, considering the standard deviations obtained.

With regard to the slight increase obtained in calculated Z_{ave} and PDI over the exposure period in the presence of SR-NOM, the absorbance remarkably also revealed higher values at 72 h, despite their standard deviation values. It was probably caused by the previously commented presence of exopolymeric exudates, which might absorb in the wavelength range selected for the UV/Vis measurements, thus interfering and increasing the absorbance measured in CeO_2 and TiO_2 dispersions.

The UV/Vis analysis was performed on a qualitative basis with the purpose of supporting the data obtained in the characterization by DLS. Therefore, it was difficult to directly compare the absorbance results with those reported in previous research (Keller et al., 2010; Li et al., 2011; Erhayem and Sohn, 2014b), which have often conducted quantitative analysis to calculate the variations in normalized NP concentrations as a function of time. Nonetheless, the absorption values found by Keller at al. (Keller et al., 2010) for CeO_2 NP dispersions were lower than that of TiO_2 NP dispersions, which is in accordance with the results obtained in the present study.

3.3. Characterization by scanning electron microscopy (SEM)

Electron microscopy has been used in previous studies to illustrate the differences in the agglomerate sizes of CeO_2 and TiO_2 NPs dispersed under various methods in algal media (Schwabe et al., 2013; Manier et al., 2013; Lin et al., 2012). The test dispersions prepared in the present study exhibited quite low polydispersity in the DLS characterization carried out. However, different agglomerate sizes were still found and it is well-known that SEM images permits only the visualization of a tiny area of the dispersions. Moreover, the preparation of SEM samples involved changes in the ultimate disposition of the nanoparticles studied. During their drying process, NOM and growth medium substances could have crystallized, and CeO_2 and TiO_2 NPs possibly formed larger agglomerates. Therefore, this disposition was not completely comparable to their state in dispersion. In spite of these uncertainties, the overall findings and insights obtained by the previous characterization were supported by SEM.

The imaging conducted (Figs. 3, 4 and S17) supported the previously mentioned fact that SR-NOM reduced the Z_{ave}, thus increasing the stability of the test dispersions. Although variations in Z_{ave} as a function of SR-NOM concentration were not substantial, increasing organic matter concentrations resulted in decreasing agglomerate sizes in most cases (Figs. 3C, 3D, 4C, 4D and S17). In addition, SEM images contributed to observe the greater stability of TiO_2 dispersions provided by SR-NOM with respect to that of CeO_2 dispersions. For instance, the reduction in the agglomerate sizes of CeO_2 NPs shown in Fig. 3 was less significant than that shown by TiO_2 NPs in Fig. 4.

The increase in the Z_{ave} after 72 h of exposure, observed mainly in CeO_2 dispersions in the presence of 20 mg/L SR-NOM was illustrated in Figs. 3C and D. As already mentioned, in the absence of SR-NOM, DLS measurements did not clearly exhibit the same trend for the test dispersions, because of considerable standard deviation. Nevertheless, the SEM imaging was useful to observe that larger agglomerates were also found after the exposure period in this case (Figs. 3A, B and 4A, B). It was reasonable, taking into account that nutrient salts in the algal growth medium and the exopolymeric exudates excreted by alga during the tests contributed to agglomeration.

The influence of SR-NOM in the variations of the PDI observed for CeO_2 and TiO_2 dispersions was also shown by the SEM characterization. TiO_2 test dispersions exhibited higher PDI with increasing concentrations of SR-NOM, illustrated by the greater variability in the size distributions in Figs. 4C and D. The opposite behavior was shown by CeO_2 test dispersions, with higher PDI in the absence of SR-NOM, observed in Figs. 3A and B.

3.4. Algae ecotoxicity studies

CeO_2 and TiO_2 NPs in the absence of SR-NOM showed considerable adverse effects even at the lowest concentrations tested (Table 4 and Fig. 5). Flocculation and clustering of NPs around *P. subcapitata* cells were observed (Supplementary Data, Fig. S18) in these dispersions, which have been previously proposed to cause artifacts in toxicity

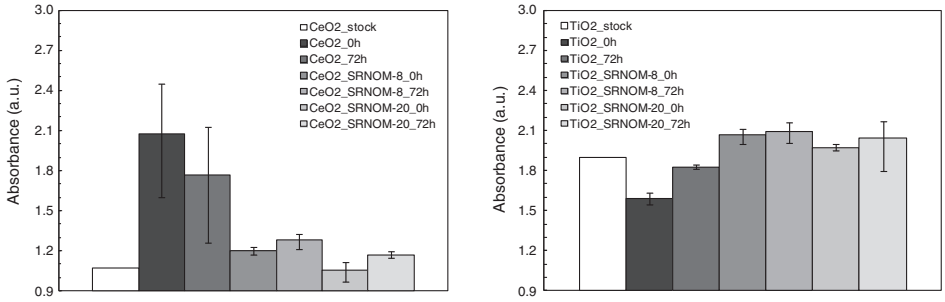

Fig. 2. Histogram comparisons of UV/vis absorbances of the 160 mg/L NP dispersions performed at 400 nm. Error bars represent standard deviation (n = 3).

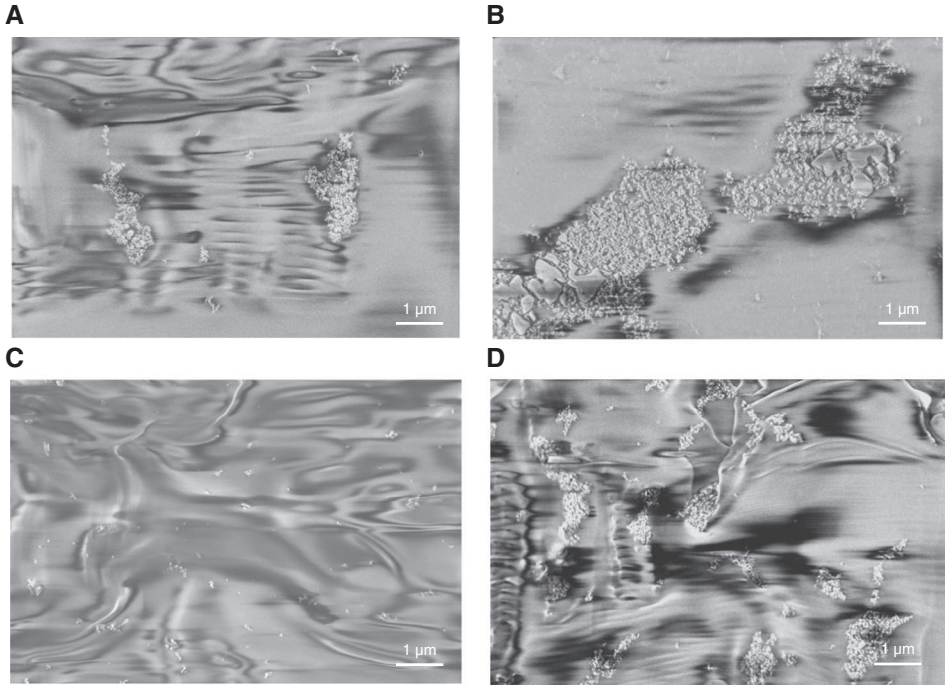

Fig. 3. SEM images of the 160 mg/L NP dispersions: (A) CeO_2 _0h, (B) CeO_2 _72h, (C) CeO_2 _SRNOM-20_0h, (D) CeO_2 _SRNOM-20_72h.

tests by a local nutrient depletion and/or shading at the cellular level (Van Hoecke et al., 2009). The 72 h-EC50 value obtained for pristine CeO_2 NPs and *P. subcapitata* in the absence of SR-NOM in the present study (1.24 mg/L) was consistent with the variable toxic effects reported by Manier et al. (4.1–6.2 mg/L) (Manier et al., 2013), Rodea-Palomares et al. (2.4–29.6 mg/L) (Rodea-Palomares et al., 2011), and Van Hoecke et al. (10.2–19.1 mg/L) (Van Hoecke et al., 2009). In the case of TiO_2 NPs, Hartmann et al. (Hartmann et al., 2010) found EC50 values of 71.1–241 mg/L, and Menard et al. (Menard et al., 2011) obtained values as low as 5.83 mg/L. The TiO_2 NPs analyzed in the present study showed an EC50 value of 0.27 mg/L, quite lower than those proposed in the literature, indicating even greater variability than in the case of CeO_2 NPs. Although the lack of stability of the dispersions in the absence of SR-NOM might be a determining factor in the reproducibility of the test results, the intrinsic physicochemical properties of CeO_2 and TiO_2 NPs also probably played an important role in their variable adverse effects. For instance, toxicity of CeO_2 NPs towards *P. subcapitata* has been found to increase with decreasing nominal

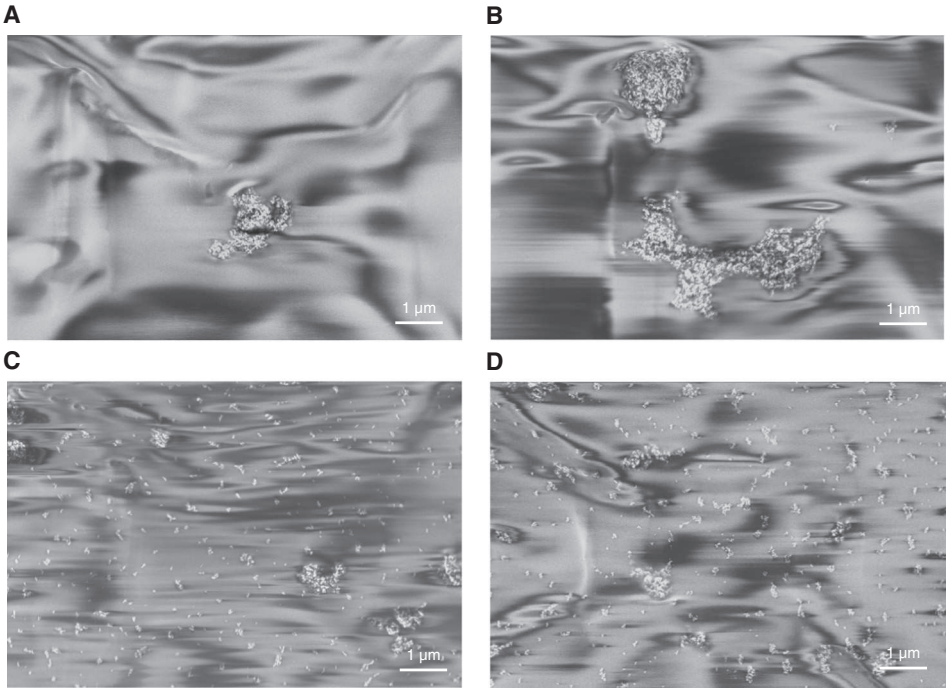

Fig. 4. SEM images of the 160 mg/L NP dispersions: (A) TiO_2 _0h, (B) TiO_2 _72h, (C) TiO_2 _SRNOM-20_0h, (D) TiO_2 _SRNOM-20_72h.

Table 4
Calculated 50% effective concentration (EC50) and 10% effective concentration (EC10) of NP dispersions (mg/L) to *Pseudokirchneriella subcapitata* during 72 h, and lower and upper 95% confidence intervals (CL) from the statistical analysis (n = 3).

Test substance	EC50 (95% CL)	EC10 (95% CL)
CeO_2 NPs	1.24 (1.07–1.43)	–
TiO_2 NPs	0.27 (0.15–0.48)	–
SR-NOM	No inhibition (increase in the growth rate)	
CeO_2 NPs + SRNOM-8	31.9 (10.2–99.5)	16.2 (4.8–54.4)
TiO_2 NPs + SRNOM-8	No inhibition (increase in the growth rate)	
CeO_2 NPs + SRNOM-20	74.3 (18.0–305.9)	38.0 (8.4–171.6)
TiO_2 NPs + SRNOM-20	No inhibition (increase in the growth rate)	

Note: The pH values at the end of the tests decreased slightly from the initial 8.2–8.3 to average values of 7.8. In the case of metals and compounds that partly ionize at a pH around the test pH, OECD Guideline 201 requires a pH drift of less than 0.5 to obtain reproducible and well defined results. Thus, the pHs were kept in the range of the validity criteria during the exposure period. The biomass in the control cultures increased exponentially by a factor corresponding to specific growth rates of 0.9 day^{-1}. This value was lower than the specified by the OECD Guideline 201, but otherwise acceptable, taking into account that one of the nutrients in the algal growth medium was removed to avoid binding on metal ions (further details provided in the Supplementary Data).

particle size (Van Hoecke et al., 2009), and Booth et al. (2015) obtained EC50 values of 0.024 mg/L for CeO_2 NPs with sizes between 4 and 10 nm.

The different effective concentration values observed between CeO_2 and TiO_2 NPs were influenced by their physicochemical features, which led to specific ecotoxicity mechanisms. Reactive oxygen species (ROS) generated by CeO_2 NPs were reported to produce a loss of the lipid peroxidation recovery and radical scavenging activity of *P. subcapitata* cells during 72 h (Booth et al., 2015). Hartmann et al. (Hartmann et al., 2010) proposed ecotoxicity mechanisms of TiO_2 NPs towards algae such as ROS generation, adhesion of NPs to algal cells and physical disruption of the cell membranes. The TiO_2 NPs tested in the current study showed higher content of impurities than that of CeO_2 NPs (Table 1), which might have also determine the lower EC50 values obtained in the absence of SR-NOM (Hartmann et al., 2010; Handy et al., 2012).

Increasing SR-NOM concentrations in the algal medium reduced toxicity and resulted in a significant increase of the 50% effective concentration (74.3 mg/L for CeO_2 NPs, no inhibition observed for TiO_2 NPs after adding 20 mg/L SR-NOM). The literature reporting EC50 values for these NPs in the presence of NOM is almost non-existent. However, Van Hoecke et al. (2011) obtained 48 h-EC20 values for CeO_2 NPs and *P. subcapitata* between 26.0 and 81.6 mg/L in the presence of organic matter concentrations similar to those used in the present study. Taking into account the different exposure times and endpoints calculated in the current research (72 h-EC50 and EC10), the herein obtained toxicity results were in the same range.

The inhibition histograms corresponding to the tests performed in the absence of organic matter (Fig. 5B, E) showed that representing

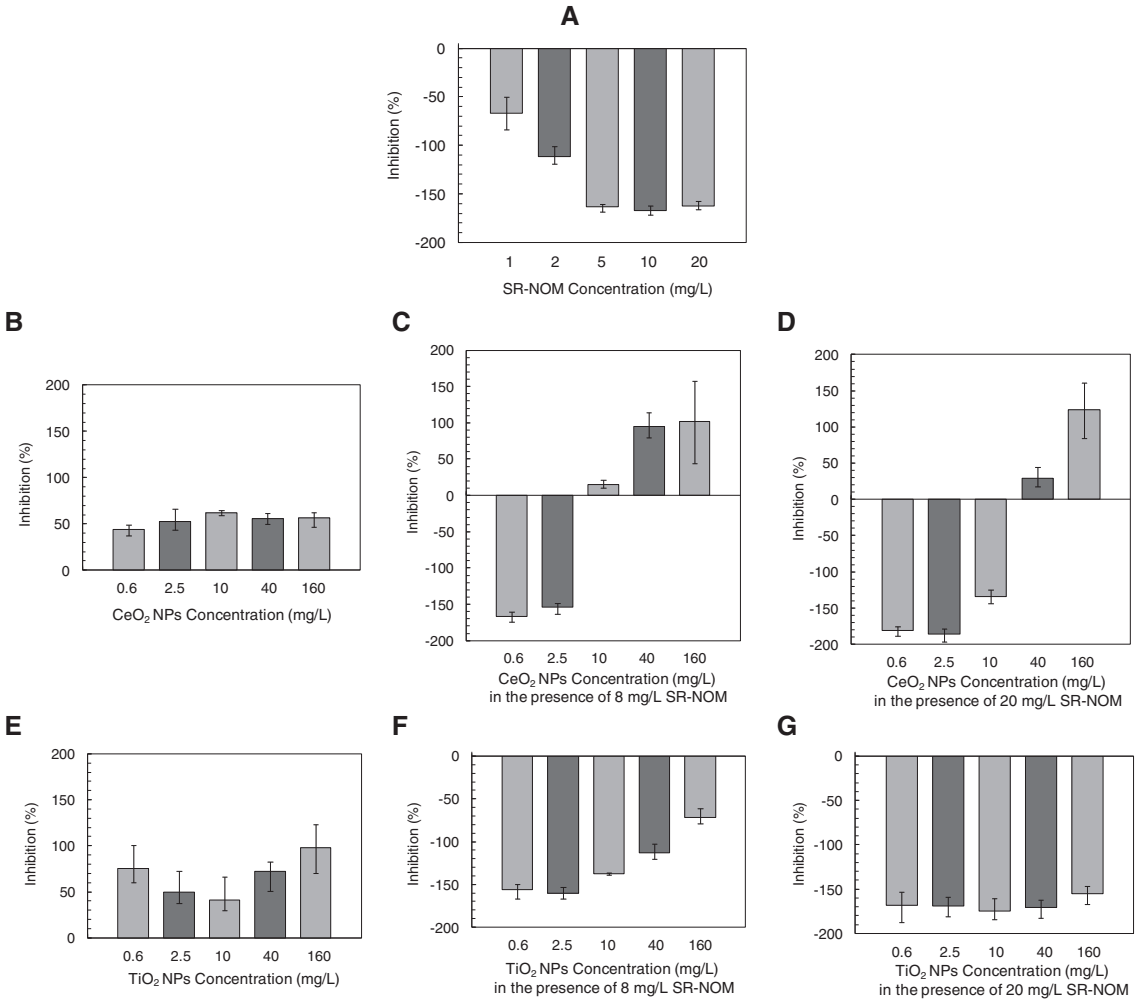

Fig. 5. Histogram comparisons of percent inhibition in average specific growth rates of *Pseudokirchneriella subcapitata* exposed to SR-NOM (A), CeO_2 and TiO_2 NPs in the absence of SR-NOM (B,E), CeO_2 and TiO_2 NPs in the presence of 8 mg/L SR-NOM (C,F) and CeO_2 and TiO_2 NPs in the presence of 20 mg/L SR-NOM (D,G), during 72 h. Error bars represent standard deviation (n = 3).

dose–response curves would have resulted in poor fits with considerable statistical spread. The severe agglomeration of NPs in the algal growth media observed (Z_{ave} ranging from 2 to 3 μm) hindered obtaining reproducible dose–response relationships, as previously reported (Hartmann et al., 2010). In contrast, the enhanced stability provided by SR-NOM led to a better reproducibility of CeO_2 and TiO_2 NPs testing, in the presence of both 8 mg/L and 20 mg/L SR-NOM (Fig. 5C, D, F, G). These histograms showed better fit of dose–response relationships, since inhibitions increased with NP concentrations. Similarly, Cupi et al. (2015) proposed that, for some NPs, the presence of NOM may be an important variable to achieve constant exposure conditions, leading to improved reproducibility of their standardized testing.

In the field of nanoecotoxicology it is assumed that stability provided by organic matter results in increased exposure of aquatic biota to NPs (Grillo et al., 2015). For instance, the toxicity of TiO_2 NPs to developing zebrafish *Danio rerio* was found to be enhanced after the addition of NOM (Yang et al., 2013). Nonetheless, this fact depends on the trophic level of the organism studied. Lin et al. (2012) reported that the presence of synthetic HA increased the negative zeta potential of TiO_2 NPs and alleviated their toxicity to the unicellular green algae *Chlorella* sp. Van Hoecke et al. (2011) also observed a significant decrease in the toxicity of CeO_2 NPs stabilized with organic matter sampled from a creek towards *P. subcapitata*. Likewise, in the present study the use of organic matter led to a better stability of the dispersed NPs during the tests and at the same time significantly attenuated their adverse effects on algal growth. The colloidal stability provided by SR-NOM to CeO_2 and TiO_2 NPs did not increase their ecotoxicity because bioavailability was influenced by other interaction mechanisms. SR-NOM might 'camouflage' the toxicity of these NPs towards algae due to mechanisms such as complexation, adsorption, electrostatic forces, and oxidation/reduction, as reported by Grillo et al. (2015). The reduction in the effects of CeO_2 and TiO_2 NPs suggested that organic matter also acted as stimulating growth factor, taking into account the increase in the growth rates observed in the tests performed independently with SR-NOM (Fig. 5A).

The NOM concentrations tested completely eliminated the toxicity of TiO_2 NPs and caused 'negative inhibitions' on algal growth (Fig. 5F, G). In the case of CeO_2 NPs, this behavior was only observed for concentrations up to 2.5 mg/L and 10 mg/L in the presence of 8 mg/L and 20 mg/L SR-NOM, respectively. The morphology and greater particle surface area of TiO_2 NPs were probably determining factors in their enhanced interactions with SR-NOM (discussed in Section 3.1), which produced a dramatic reduction in their toxicity compared to that of CeO_2 NPs. Adverse effects ('positive inhibitions') for TiO_2 NPs might be expected by reducing significantly the amount of NOM in dispersions. The correlation represented in Fig. S19 (Supplementary Data) provided some guidance on this concentration, which should be around 2 mg/L SR-NOM or even lower, depending on the TiO_2 NP concentration. However, the representative amounts of organic carbon in freshwater environments for *P. subcapitata* are higher than those corresponding to this concentration of SR-NOM (Cupi et al., 2015; Thurman, 1985). Furthermore, the expected concentrations of MNMs in natural waters of approximately 1 to 100 μg/L (Klaine et al., 2008) are in the range causing negative inhibitions in the presence of the lowest concentration of SR-NOM tested (up to 2.5 mg/L, Fig. 5C, and up to 160 mg/L, Fig. 5F). Therefore, the 'camouflage' of the ecotoxicity of CeO_2 and TiO_2 NPs towards algae might occur even for small amounts of NOM and the highest predicted amounts of NPs in aquatic systems. Taking into account the herein obtained results and the stability provided by SR-NOM to metal oxide NPs in algae medium even in the long term (Quik et al., 2010), it seems reasonable to introduce SR-NOM into standardized testing methods to assess the ecotoxicity of CeO_2 and TiO_2 NPs towards green microalgae. Further research is needed to analyze its suitability in the ecotoxicological assessment of other nanomaterials, and also to select the specific SR-NOM concentration (or concentration ranges) used in the tests.

4. Conclusions

The present study has demonstrated the usefulness of SR-NOM in the assessment of the agglomeration kinetics and ecotoxicity of CeO_2 and TiO_2 NPs towards green microalgae. SR-NOM alleviated their adverse effects on *P. subcapitata* growth, completely in the case of TiO_2 NPs and partially in the case of CeO_2 NPs. Previous studies have evidenced this behavior for other algal species and types of NOM. Furthermore, SR-NOM increased significantly the stability of the NPs in dispersions, which led to a better reproducibility of the toxicity test results. The electrophoretic mobility provided by SR-NOM to CeO_2 and TiO_2 NPs was similar to that previously reported in various river and groundwaters. Therefore, SR-NOM might be a representative sample of what is found in many different ecosystems, thus fulfilling the simulation of realistic environments required for the standardized ecotoxicological assessment of these NPs. The 'camouflage' of the effects of CeO_2 and TiO_2 NPs on algal cells might take place even for small amounts of SR-NOM and the highest predicted amounts of NPs in natural waters.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.10.137.

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SUPPLEMENTARY DATA

Towards the standardization of nanoecotoxicity testing: natural organic matter 'camouflages' the adverse effects of TiO_2 and CeO_2 nanoparticles on green microalgae

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MATERIALS AND METHODS

Preparation of NPs dispersions

Calculation of the total amount of energy delivered by the sonicator probe from calorimetry

A 600 mL borosilicate glass beaker was filled with 500 mL thermally equilibrated Milli-Q water. Its temperature and mass were measured with an uncertainty of ±0.1 ºC and ±0.1 g, respectively. The beaker was placed in the sonicator chamber and the tip was immersed to a position 2.5 cm below the liquid surface. The temperature probe was mounted (using a clamp) at 2.5 cm depth and 1 cm away from the sonicator probe. The sonicator output selected was 20% amplitude (considering previous dispersion tests carried out in our laboratory), operating in continuous mode. The temperature increase of the water was recorded for 6.5 minutes with a time resolution of 30 seconds.

The calculation of the delivered acoustic energy was performed obtaining the best linear fit (R^2>0.990) between the measured temperature and time using least squares regression. The effective delivered power was determined using the following equation:

(S1)

—

where P is the delivered acoustic power (W), dT/dt is the slope of the regression curve, M is the mass of liquid (g), and C_p is the specific heat of the liquid ($J \cdot g^{-1} \cdot ºC^{-1}$).

The effective delivered acoustic power (P) was 11.76 W. The linear fits between the measured temperature as function of time using least squares regression are represented in Figure S1.

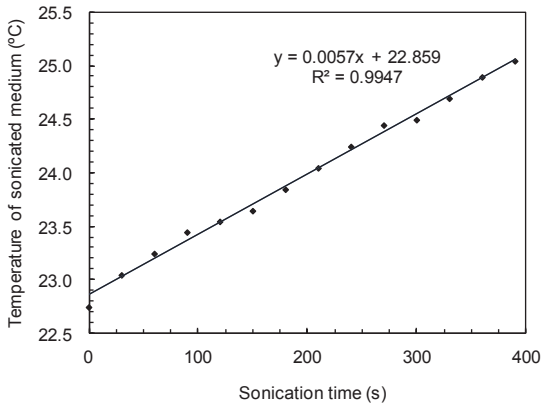

Figure S1. Linear fits between the measured temperature as function of time sonicator probe.

The total amount of energy delivered was obtained considering the applied power and also the total amount of time that the dispersion was subjected to the ultrasonic treatment.

(S2)

where E is the total amount of energy (J), P is the delivered acoustic power (W) and t is the total amount of time (s).

Considering the sonication time of 12 min (selected taking into account previous dispersion tests carried out in our laboratory), the total amount of energy delivered (E) was 8.467 KJ. The acoustic energy delivered by the probe enabled to obtain agglomerate sizes as near as possible to the nanometric range, thus optimizing the preparation of the dispersions. It was important to consider that the actual volumes and temperatures of NPs dispersions were different from that used in the calculation of the energy delivered by the sonication methods. However, this aspect was noted in the calorimetric method [1], since it was simply intended to allow the reporting and transference of sonication power levels between users, but not to measure the actual fraction of power utilized for powder disruption under specific dispersion conditions.

Algae ecotoxicity studies

Preparation of the OECD *P. subcapitata* growth medium

The algal growth medium was prepared by adding an appropriate volume of the stock solutions 1-4 to sterile ultrapure water. The stock solutions of nutrients were prepared according to the Table S1.

Table S1. Concentration of nutrients in *Pseudokirchneriella subcapitata* medium.

Stock solution	Nutrient	Concentration in stock solution	Final concentration in test solution
1: macro nutrients	NH_4Cl	1.5 g/L	15 mg/L
	$MgCl_2 \cdot 6H_2O$	1.2 g/L	12 mg/L
	$CaCl_2 \cdot 2H_2O$	1.8 g/L	18 mg/L
	$MgSO_4 \cdot 7H_2O$	1.5 g/L	15 mg/L
	KH_2PO_4	0.16 g/L	1.6 mg/L
2: Fe-EDTA[a]	$FeCl_3 \cdot 6H_2O$	64 mg/L	64 µg/L
3: trace elements	H_3BO_3	185 mg/L	185 µg/L
	$MnCl_2 \cdot 4H_2O$	415 mg/L	415 µg/L
	$ZnCl_2$	3 mg/L	3 µg/L
	$CoCl_2 \cdot 6H_2O$	1.5 mg/L	1.5 µg/L
	$CuCl_2 \cdot 2H_2O$	0.01 mg/L	0.01 µg/L
	$Na_2MoO_4 \cdot 2H_2O$	7 mg/L	7 µg/L
4: bicarbonate	$NaHCO_3$	50 g/L	50 mg/L

[a] $Na_2EDTA \cdot 2H_2O$ was removed to avoid binding on metal ions.

The stock solutions 2 and 4 were sterilized by membrane filtration (mean pore diameter 0.2 μm), and stock solutions 1 and 3 were sterilized by autoclaving (120 °C, 15 min). The solutions were stored in the dark at 4 °C. Algal growth medium was prepared by adding 10 mL of stock solution 1 and 1 mL of stock solution 2, 3 and 4 into a 1 L volumetric flask, and then filling up to 1000 mL with sterilized ultrapure water. The pH was adjusted to 8.3, with either 1 M HCl or 1 M NaOH.

Additional information on the culturing conditions

Pseudokirchneriella subcapitata stock cultures were maintained on sloped agar tubes and transferred to fresh agar at least once every two months. In order to adapt the algae to the test conditions and ensure that they were in the exponential growth phase when used in the tests, an inoculum culture was prepared in the OECD growth medium 3 days before the start of the test. The initial biomass concentration in the inoculums culture was adjusted to $5x10^5$ cells/mL to obtain a concentration of $5x10^3$ cells/mL in the volume of the test dispersions (100 mL).

Algae were grown under sterile conditions during the tests, using an orbital shaker (GFL, 3020 model) at 65 rpm and 23 ± 2 °C. Continuous illumination of 80 ± 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ (measured in the wavelength range of 400-700 nm) was provided by cool white fluorescent tubes about 30 cm distance from the position of the cultures. The light intensity was maintained within ±15% from the average over the incubation area. In addition, the position of each flask in the incubator was changed every 24 h in order to compensate any lack of uniformity in the illumination system.

Chlorophyll-a extractions and fluorescence measurements for algal growth determination

Extracted chlorophyll allowed deriving the biomass concentrations in the presence of NPs, which interfere with measurements of culture density normally made by optical absorbance. The particulates and cell debris were settled to the bottom of the tubes, whilst the chlorophyll remained in solution and was measured fluorometrically.

Samples of 1 mL from each flask containing the test cultures were extracted in a foil-wrapped screw-capped polypropylene test tube. Then, 0.1 mL of 1.5 mg/L Locust Bean Gum (Sigma-Aldrich) suspension in ultrapure water, and 4.4 ml acetone (Scharlab, HPLC grade) with $MgCO_3$, were added. The tubes were capped and inverted several times to mix, and placed in a dark cupboard at room temperature (22 ± 1°C) for 1-7 days. The samples were not exposed to bright light or air to avoid oxidative and photochemical destruction, since chlorophyll is sensitive to light and oxygen, especially when it is extracted. Homogenization of the samples was carried out to increase the extraction efficiency.

The fluorescence of the samples was determined in arbitrary units on a microplate reader (FLUOstar OPTIMA, BMG-LABTECH, Ortenberg, Germany) with an excitation wavelength of 430 nm and a measured emission wavelength of 670 nm. Measurements were performed after 24 hours extraction at room temperature and again 7 days later to check that they remained stable for that period. Fluorescence figures were corrected for background fluorescence measured on solvents mixed with algal growth medium. The needed sub-sample volume was 350 µL in 96-well Polypropylene black microplates.

A ten-point linear calibration curve (see Figure S2) was performed to obtain the algal biomass values from fluorescence measurements. A single algal culture of 5×10^5 cells/mL was obtained and a tenfold dilution series (3×10^3 to 5×10^5 cells/mL) was prepared in 10 mL vials. Three replicates from each cell density were extracted to carry out the fluorescence measurements, and the corresponding standard curves (log cells/mL vs. log fluorescence) were represented.

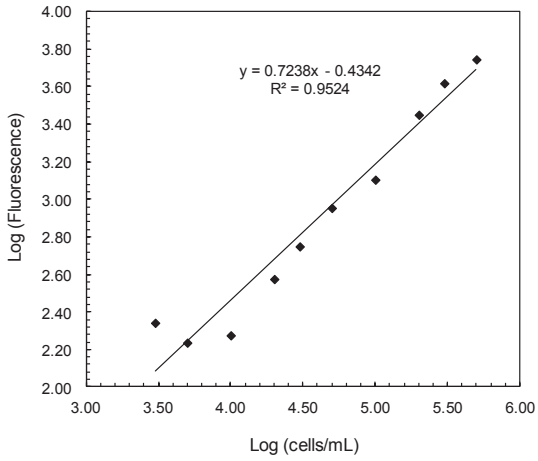

Figure S2. Calibration curve obtained from chlorophyll fluorescence in different algal concentrations (3×10^3 to 5×10^5 cells/mL). The straight lines are linear least-squares fit to the data. Excitation = 430 nm. Emission = 670 nm.

Characterization of NPs dispersions

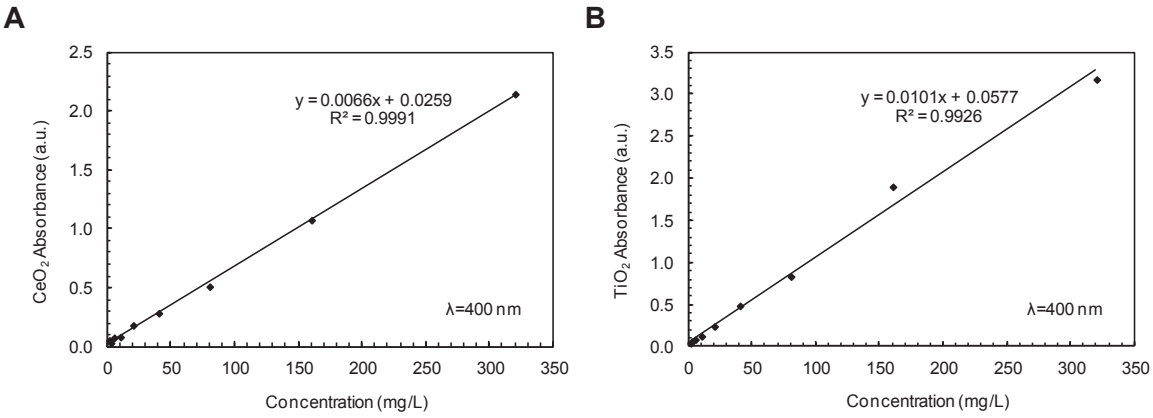

Figure S3. Calibration curves obtained from UV/vis absorbance of CeO_2 (**A**) and TiO_2 (**B**) NPs dispersions in Milli-Q water, based on several concentrations (1.25 to 320 mg/L). The straight lines are linear least-squares fit to the data.

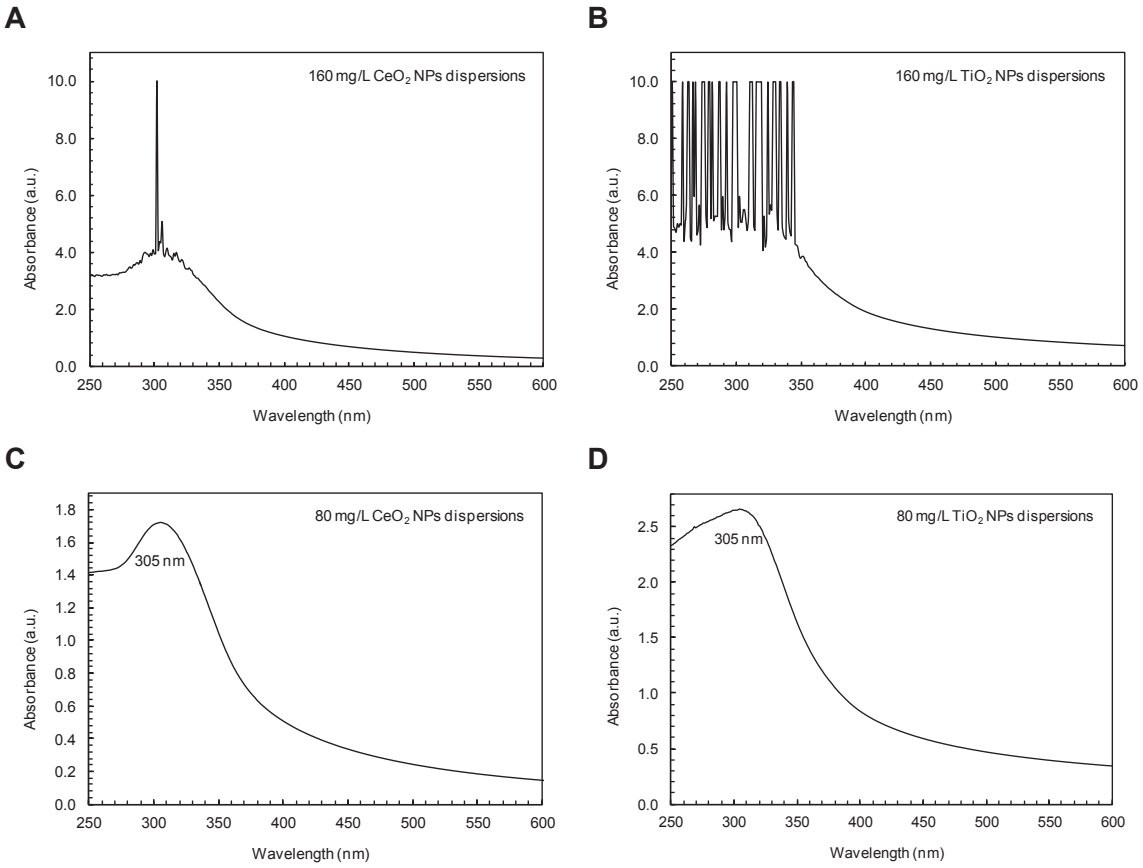

Figure S4. UV/vis spectra of the CeO_2 and TiO_2 test dispersions. Saturation of the spectrophotometer was reached near the absorbance peaks of the 160 mg/L NPs dispersions (**A, B**). Therefore, they were diluted to 80 mg/L (**C, D**) to observe exactly these peaks (approximately at 305 nm).

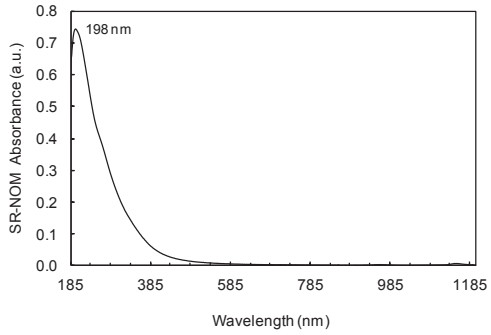

Figure S5. UV/vis spectra of 20 mg/L SR-NOM in Milli-Q water.

RESULTS AND DISCUSSION

Characterization by DLS

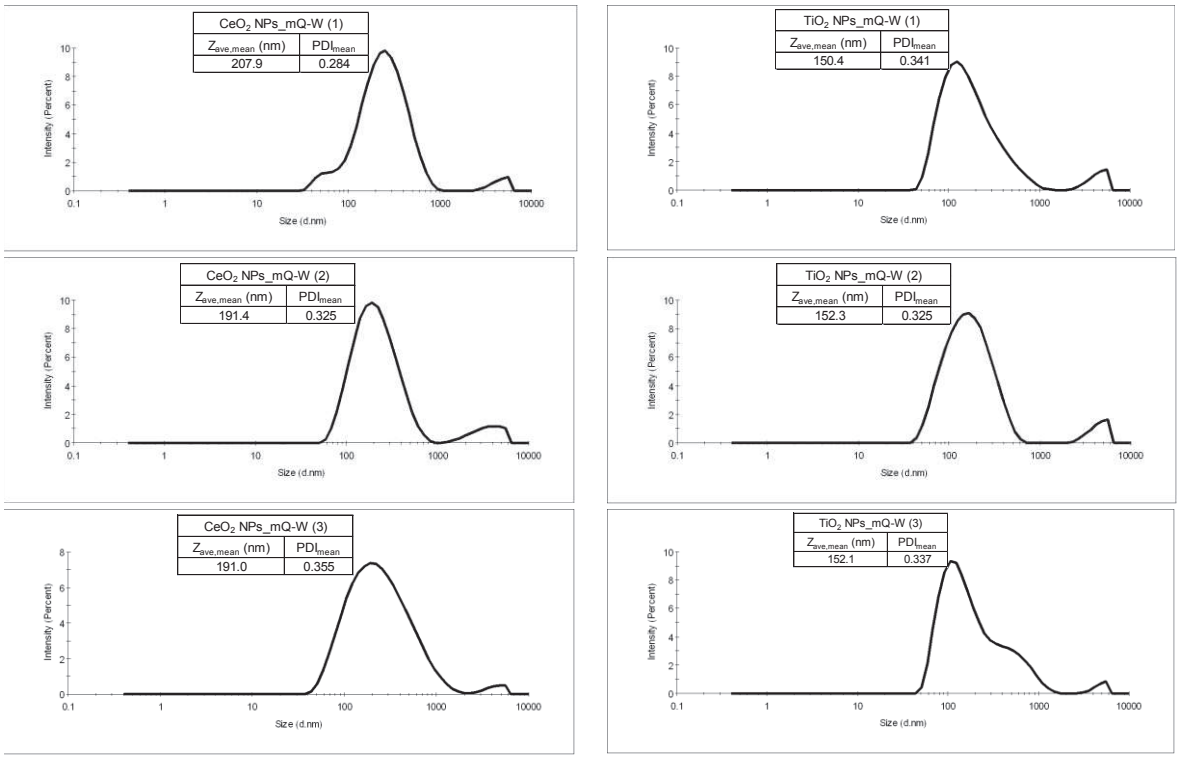

Figure S6. Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in stock dispersions. Measurements correspond to three test replicates.

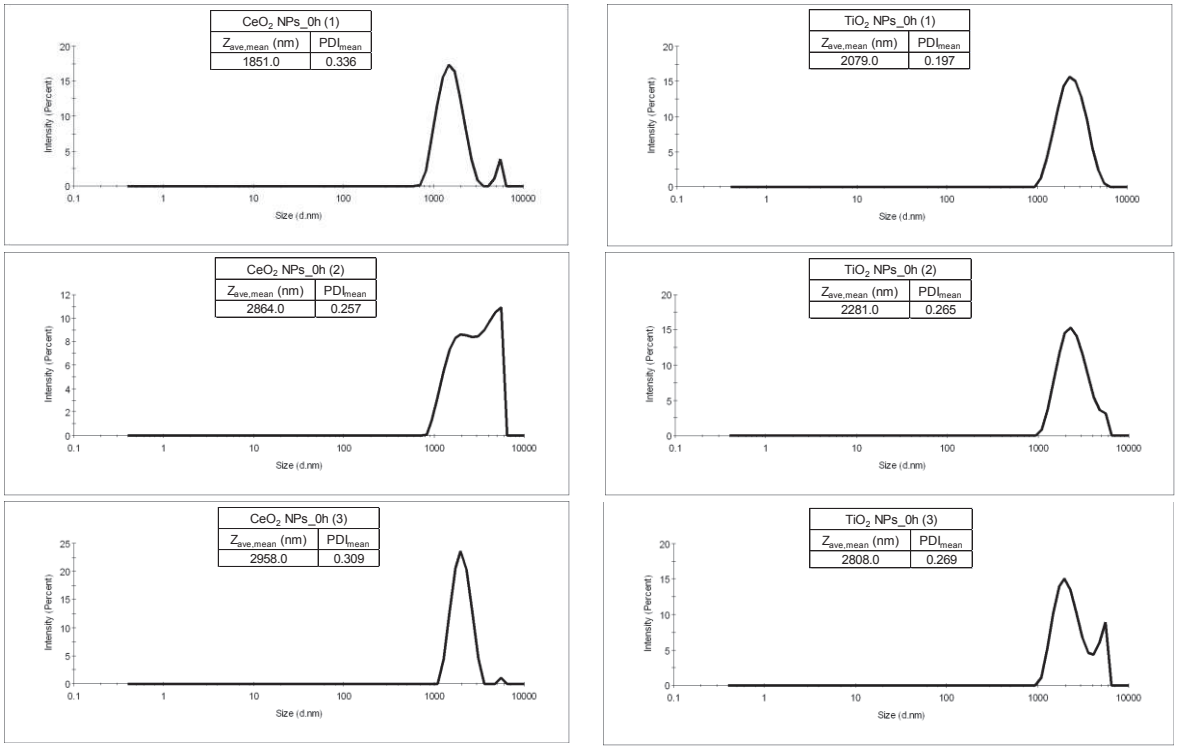

Figure S7. Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions in the absence of SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

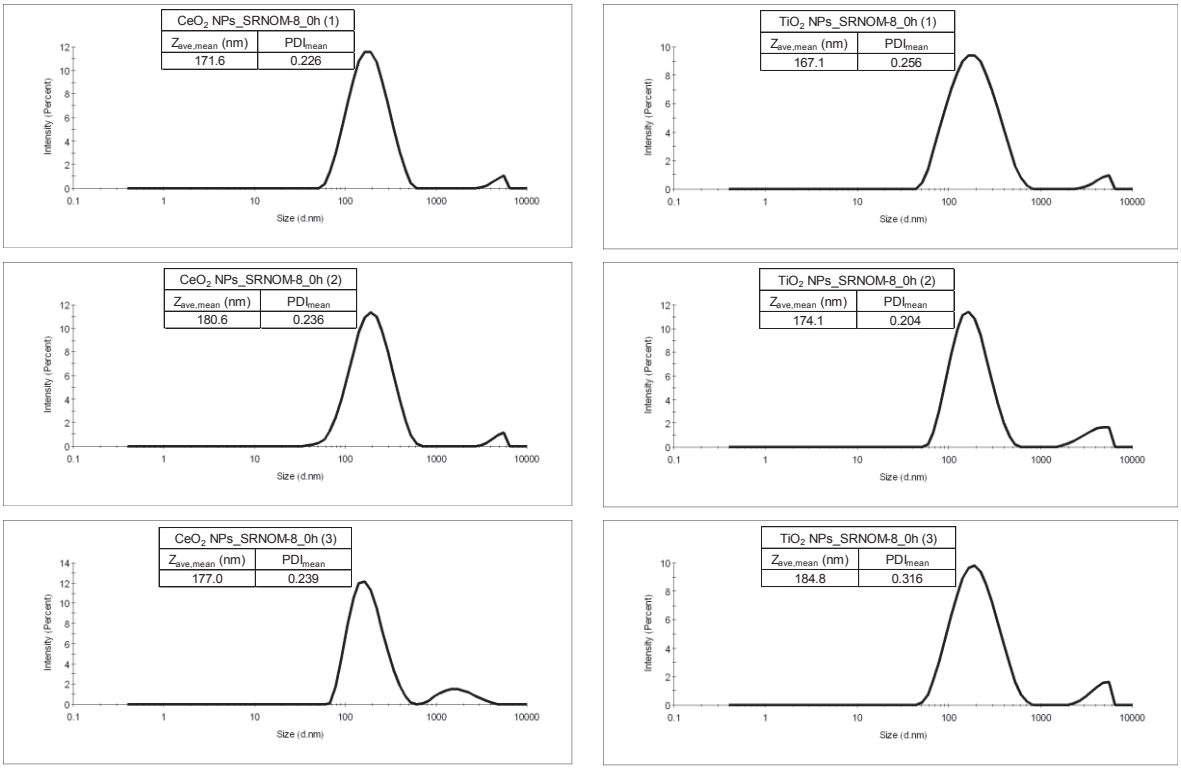

Figure S8. Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 8 mg/L SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

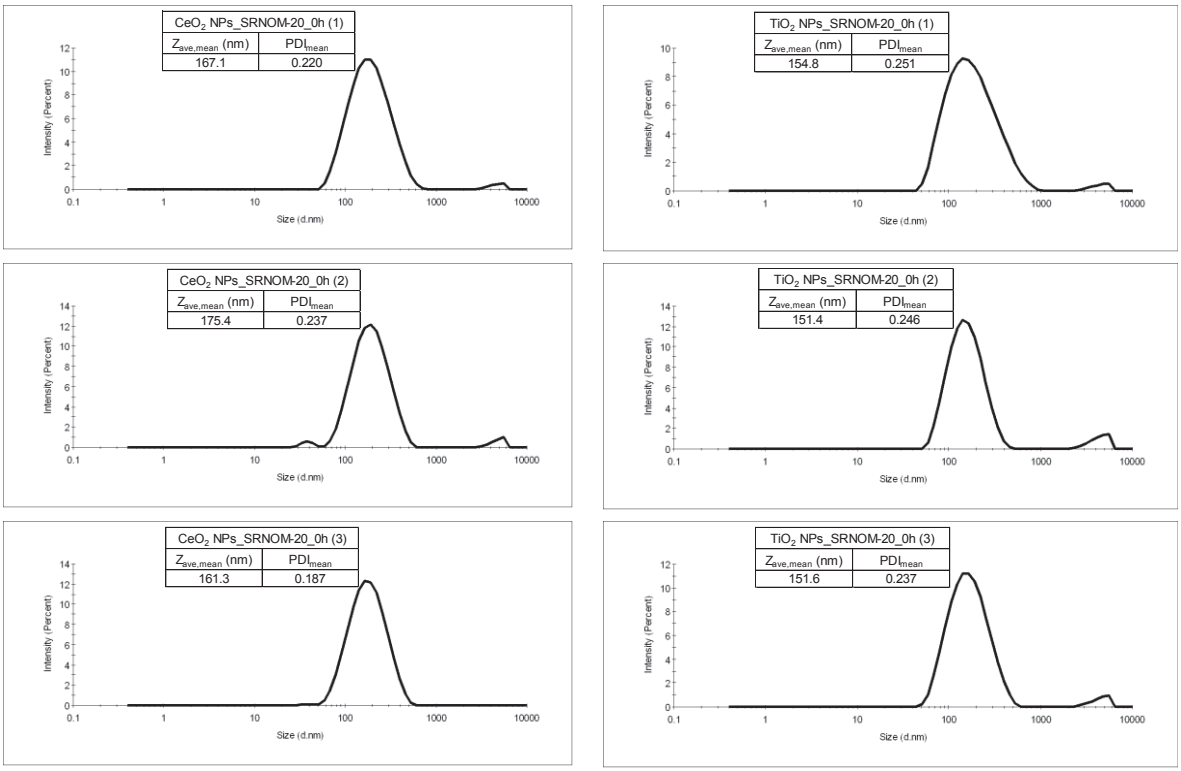

Figure S9. Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 20 mg/L SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

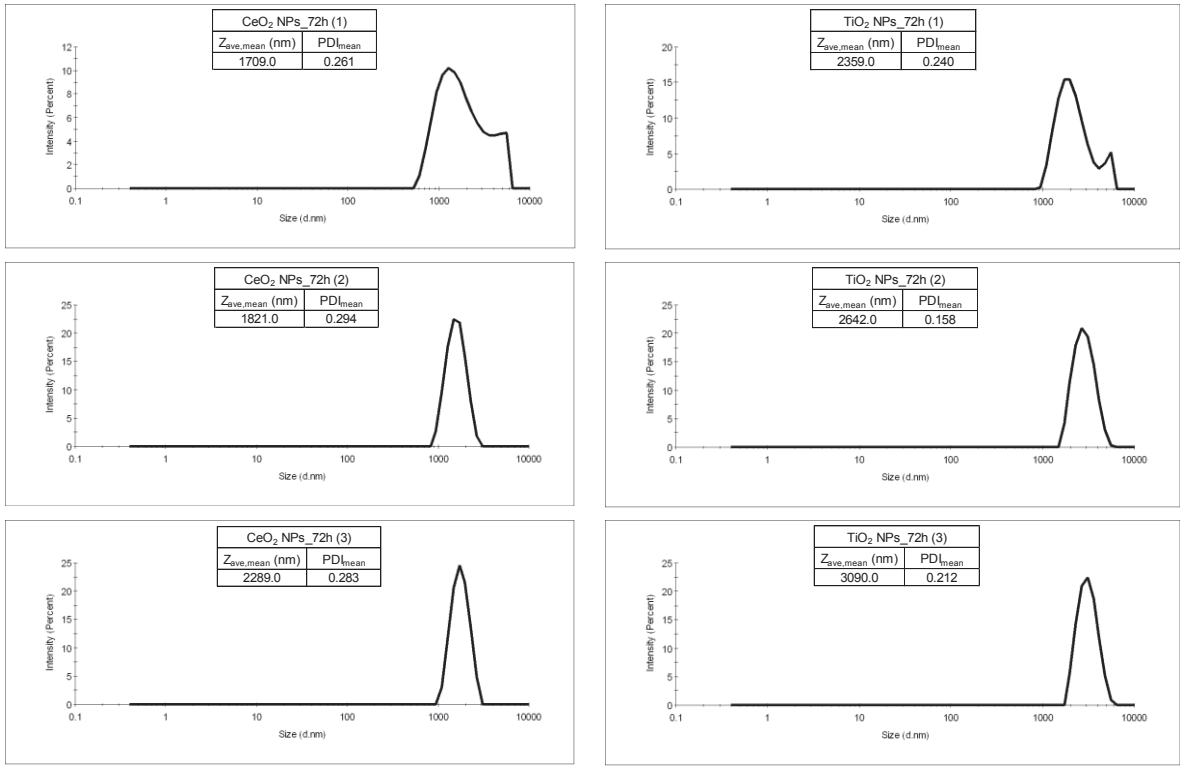

Figure S10. Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions in the absence of SR-NOM at the end of the tests. Measurements correspond to three test replicates.

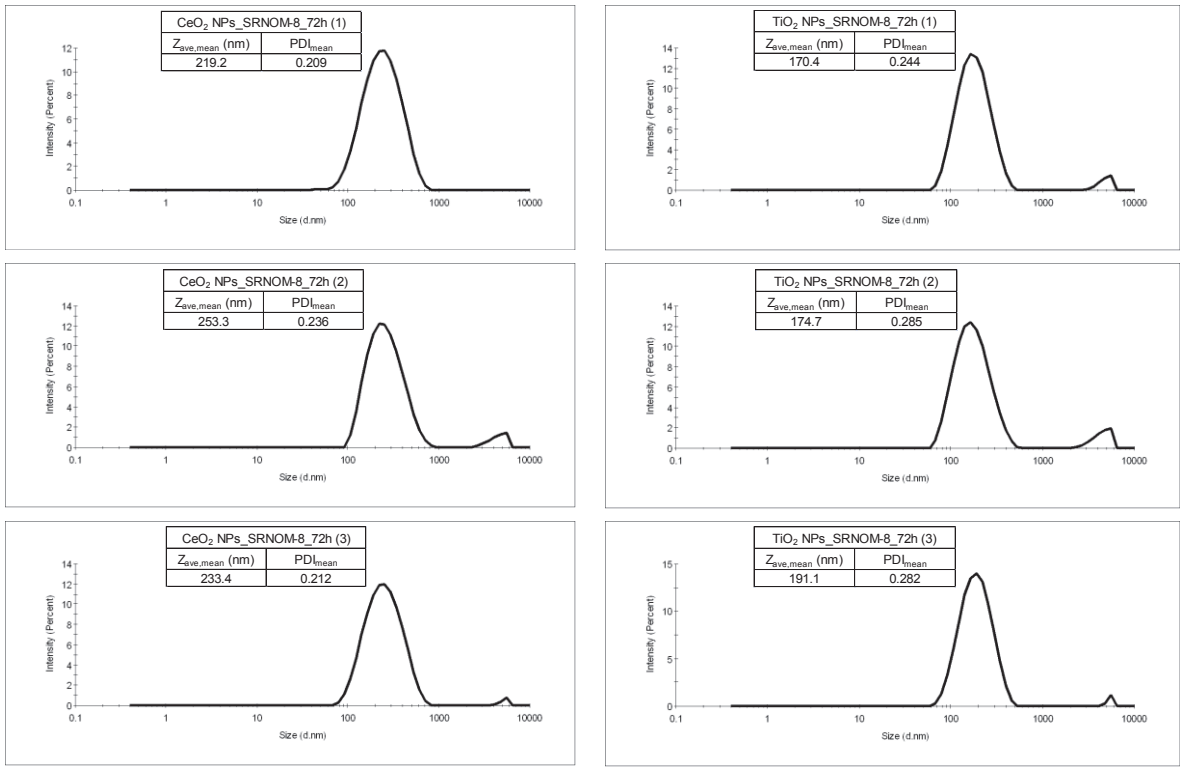

Figure S11. Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 8 mg/L SR-NOM at the end of the tests. Measurements correspond to three test replicates.

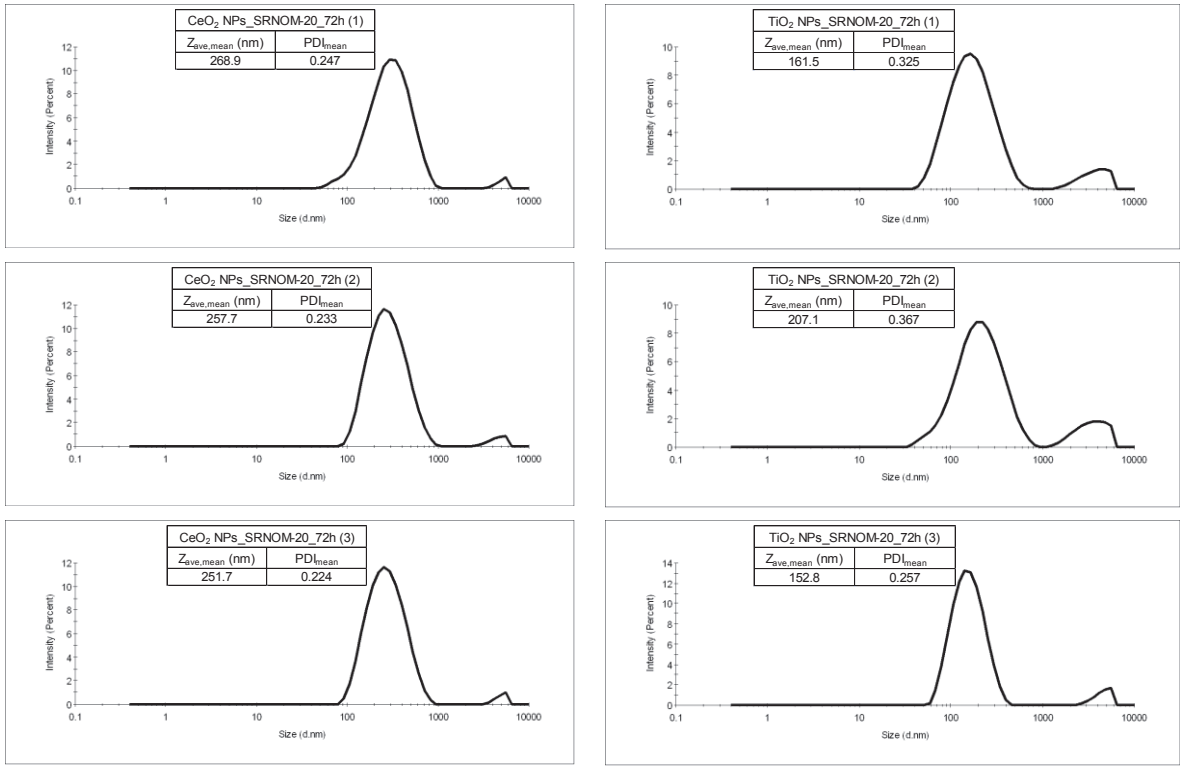

Figure S12. Size distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 20 mg/L SR-NOM at the end of the tests. Measurements correspond to three test replicates.

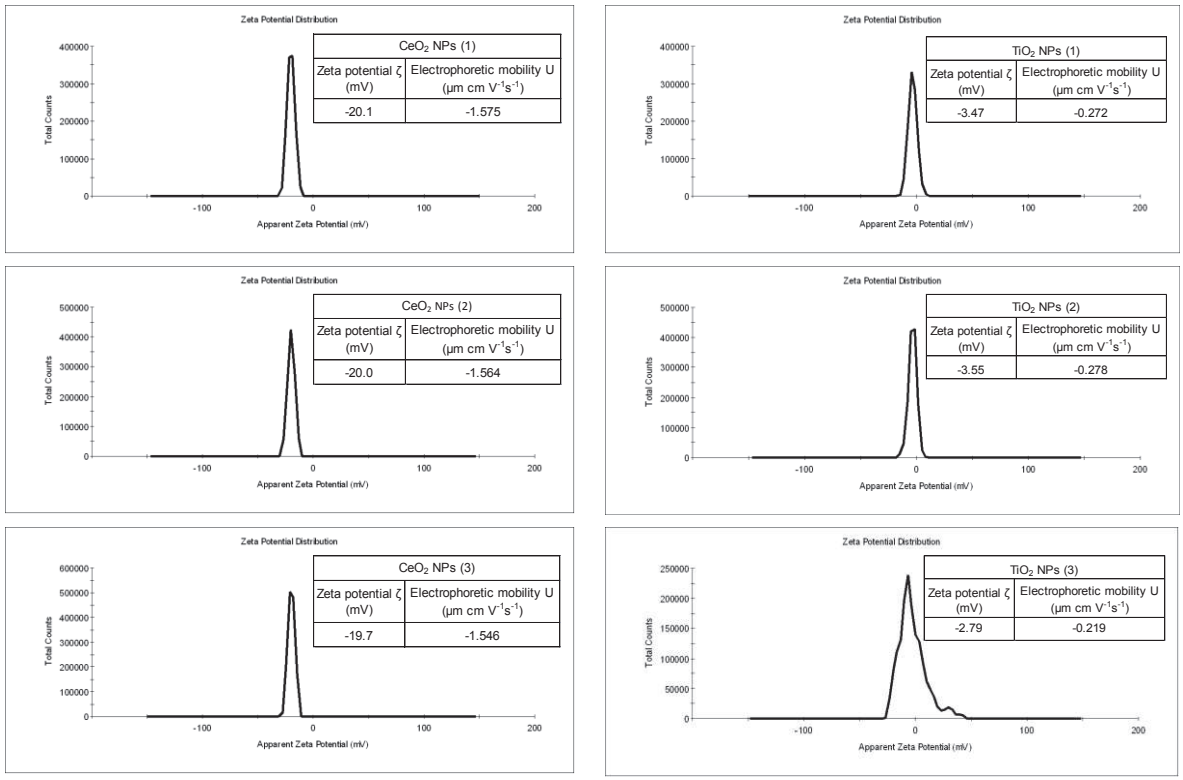

Figure S13. Zeta potential distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions in the absence of SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

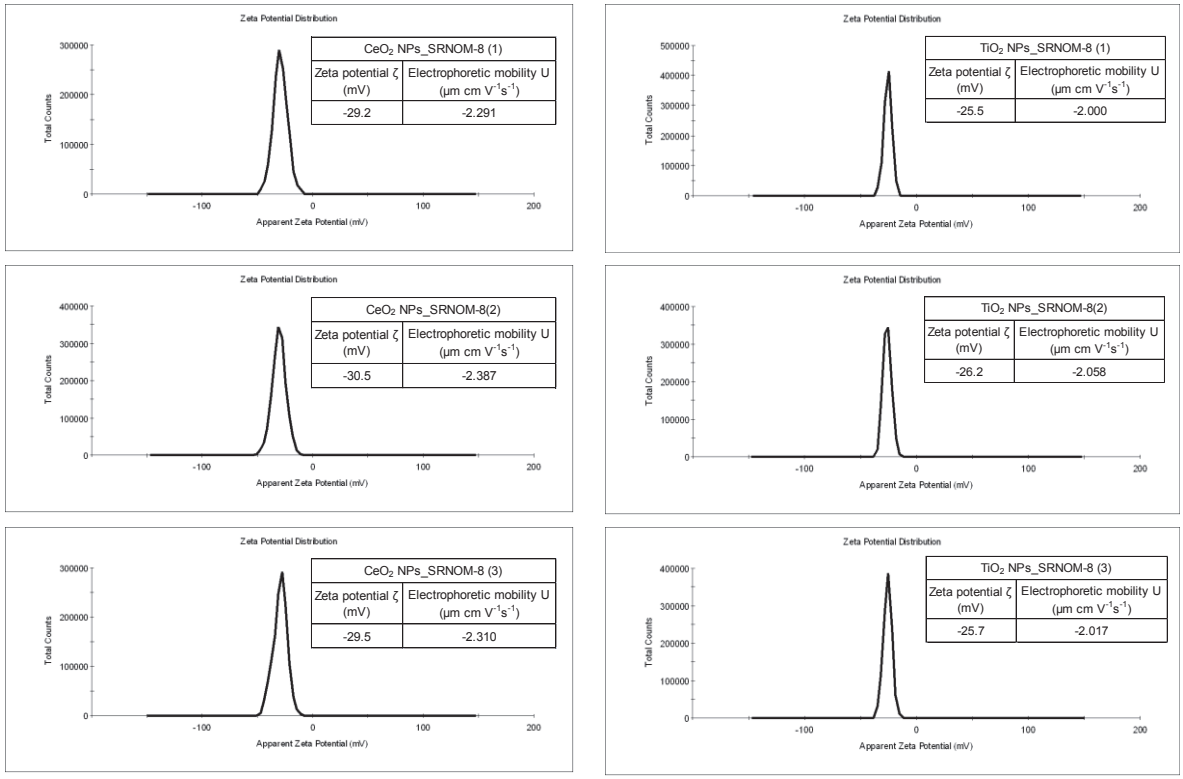

Figure S14. Zeta potential distributions by intensity of CeO_2 and TiO_2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 8 mg/L SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

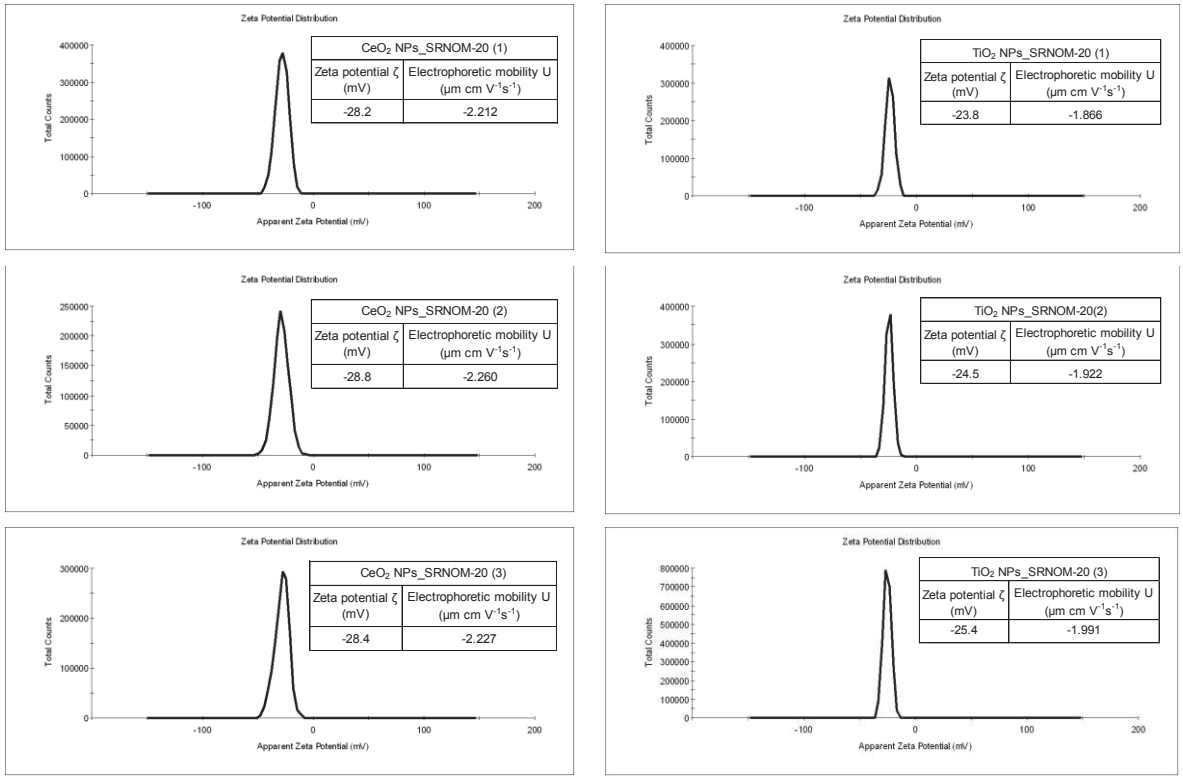

Figure S15. Zeta potential distributions by intensity of CeO2 and TiO2 NPs agglomerates in 160 mg/L NPs test dispersions prepared with 20 mg/L SR-NOM at the beginning of the tests. Measurements correspond to three test replicates.

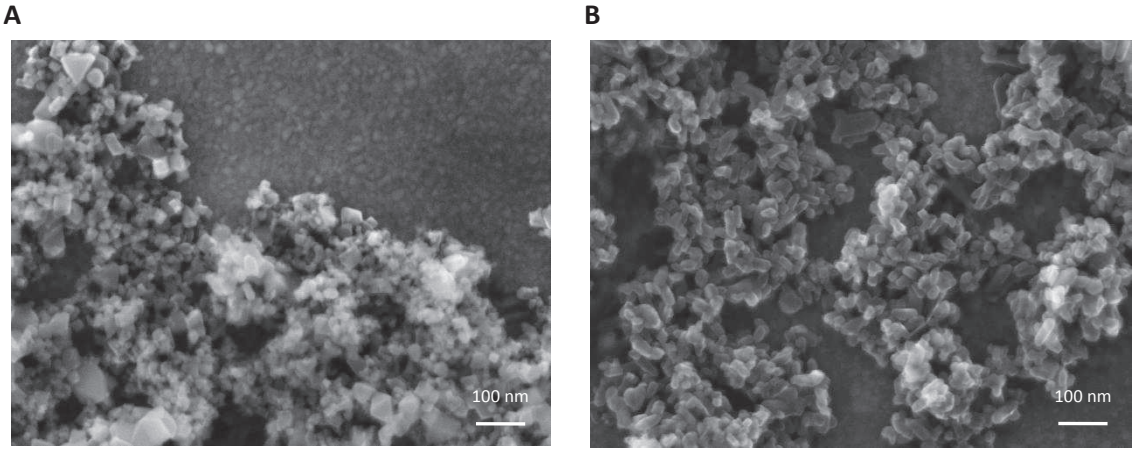

Figure S16. Field-emission scanning electron microscopic images obtained with a JEOL apparatus (JSM-7000F model) of: (A) CeO_2 NPs stock dispersions and (B) TiO_2 NPs stock dispersions.

Characterization by scanning electron microscopy (SEM)

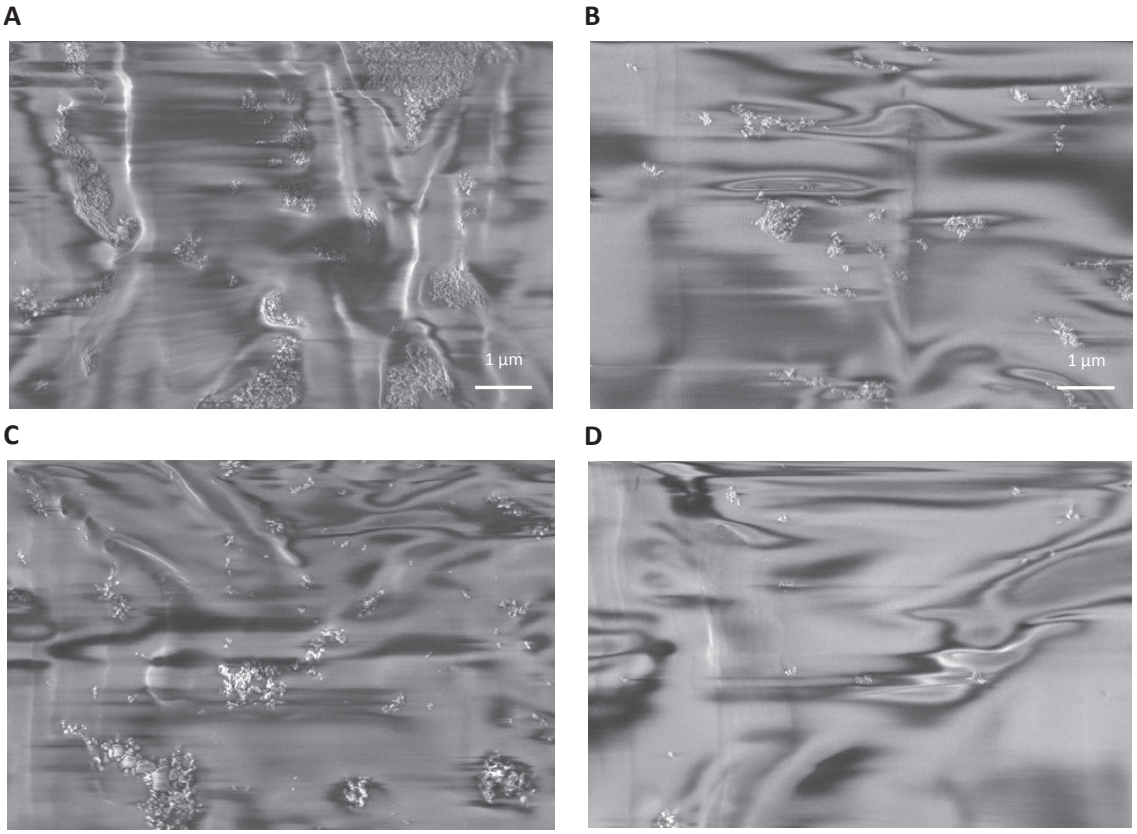

Figure S17. SEM images of the 160 mg/L NPs dispersions: (**A**) CeO_2 _SRNOM-8_0h, (**B**) CeO_2 _SRNOM-8_72h, (**C**) TiO_2 _SRNOM-8_0h, (**D**) TiO_2 _SRNOM-8_72h

Algae ecotoxicity studies

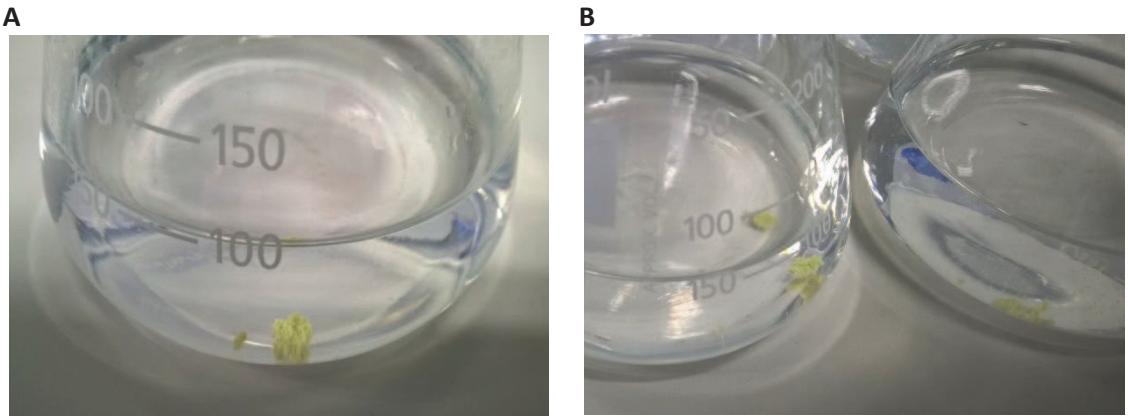

Figure S18. Clustering of TiO_2 NPs (**A**) and CeO_2 NPs (**B**) with algal cells at the end of the ecotoxicity tests in the absence of SR-NOM, for 10 mg/L NPs dispersions.

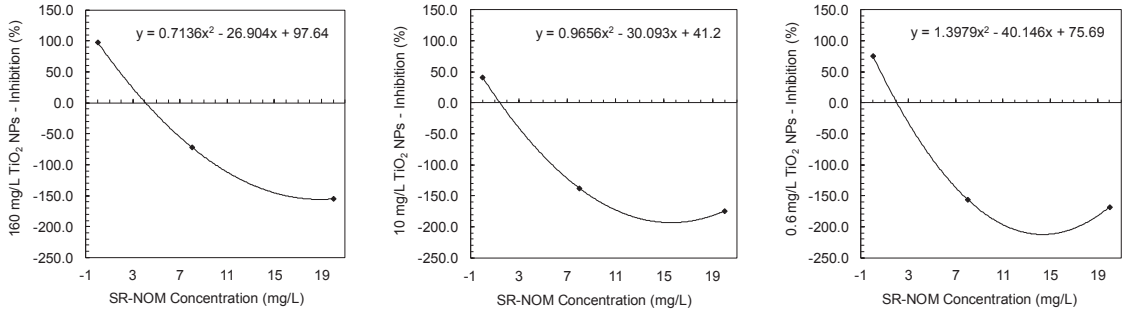

Figure S19. Correlation curves (fitted to a polynomial form, degree 2) between inhibitions produced by TiO_2 NPs test dispersions and variable concentrations of SR-NOM.

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Nanotechnology, the science of manipulating the physicochemical properties of materials on the atomic and molecular level, provides innovative solutions to various scientific disciplines. The potential and real applications of manufactured nanomaterials (MNMs) in physics, chemistry, information technology, or medicine are growing exponentially. However, their novel features have led to questions about physical, health and environmental risks.

Natural resources and biodiversity constitute fundamental assets for the survival of all kind of live in the Earth. One of the most important pathways for the entrance of MNMs and their transfer throughout the food web is represented by aquatic organisms, but the lack of standardized assessment protocols has led to contradictory toxicity results in natural waters.

The present study was aimed at defining test methods to overcome the limitations of the toxicological assessment of MNMs in aquatic ecosystems. Organisms of different trophic levels, selected in terms of cost, ecological relevance, reproducibility and sensitivity, were exposed to multiwalled carbon nanotubes (MWCNTs) and TiO_2 and CeO_2 nanoparticles, currently included in the prioritization lists of relevant reference materials worldwide.

The standardization approach of the current work was also addressed through the optimization of the energy delivered to the MNMs during the preparation of the aqueous dispersions, and the selection of a reference natural organic matter to conduct the exposures in environmentally realistic conditions. The methodologies proposed have improved the reproducibility of the toxicity test results. In addition, the influence of the test materials and methods in the colloidal stability of MNMs and their adverse effects towards aquatic organisms has been demonstrated.

The EU-FP7 NANoREG project, aimed to give an answer to regulators and legislators on Environmental, Health and Safety aspects of MNMs, has provided an essential framework for the present study.

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SEVENTH FRAMEWORK PROGRAMME

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Research Alliance

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