Environmental Impact Assessment of Nanomaterials in the Aquatic Environment

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Abstract

Nanotechnology is one of the fastest-growing technologies in the world. Silver nanomaterials (Ag NMs) and multi walled carbon nanotubes (MWCNTs) are the most commonly used NMs in consumer products for their antimicrobial properties and for their unique mechanical and electrical properties. Their use can lead to contamination of the aquatic environment. In this research study, the freshwater microalga *Raphidocelis subcapitata* was used to identify the impacts of NMs on the aquatic environment. The aim of this study was to carry out an evaluation of the toxicity of Ag NMs and MWCNTs via acute exposure to *R. subcapitata*. Moreover, two types of sonication – bath and probe – were used for comparison, to evaluate how suspension methods may affect NMs. DLS and TEM were used for NM characterisation. The probe-sonication dispersion method was a more effective process to prepare Ag NM suspensions since they were dispersed better, compared to bath sonication. However, the probe sonication was not an appropriate way for MWCNTs preparation.

The OECD 201 test guideline was used to evaluate the toxicity of representative NM at 24, 48 and 72 h. Effects were assessed by using the following four different methods: chlorophyll extraction; optical density; protein content, and photosynthetic activity. The impact of the sonication method (bath or probe) used to prepare NM dispersions on the toxicity to algae was also evaluated. Overall outputs, based on chlorophyll extraction, demonstrated a high sensitivity of *R. subcapitata* exposed to Ag NMs and MWCNTs, which led to an increase in growth inhibition with increasing concentration of the tested materials. The chlorophyll extraction method was the best method for evaluating the algal biomass with a clear concentration response and no interference of the NMs with the method.

Evidence for internalisation of Ag NMs into the *R. subcapitata* cells was shown for Ag NM dispersed using the probe-sonication methods, lower levels of Ag NM uptake were observed for bath sonicated Ag NMs. Finally, the role of oxidative stress in NM toxicity to algae was investigated. This highlighted an enhanced anti-oxidant defence system of *R. subcapitata* when subjected to NM suspensions prepared by probe sonication, as compared to bath sonication. In summary, this research has shown that Ag NMs are more toxic to *R. subcapitata* than MWCNTs and that the sonication method used to suspend the NMs affects toxicity, as Ag NMs show higher toxicity when dispersed by bath sonication, whereas MWCNTs are more toxic when dispersed

via probe sonication. It is of the utmost importance to consider carefully NM dispersion approaches, particularly when devising standard methods. It is also very important to understand how dispersant NMs may be affected by different dispersion methods.

DEDICATION

This thesis is dedicated to my parents,
My daughters Joud, Fajr, Farah and Raghad,
My beloved wife Mona,
and my beloved sisters and brothers

For their endless loves, prayers, supports
and encouragements sustained me throughout my life.

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List of Abbreviation

AAP	Algal assay procedure
AFM	Atomic force microscopy
Ag	Silver
Ag^{+}	Silver ion
$AgNO_3$	Silver nitrate
Ag NMs	Silver nanomaterials
Al_2O_3 NMs	Aluminium oxide nanomaterials
ANOVA	Analysis of variance
Au	Gold
BBM	Bold's basal medium
BSA	Bovine Serum Albumin
C60	Fullerene
$[C_2H_4O]_nC_{14}H_{22}O$	Triton X-100
$[C_6H_9NO]_n$	Tetrahydrofuran (THF)
C_4H_8O	Poly(vinylpyrrolidone) (PVP)
$C_{12}H_{25}O_4SNa$	Sodium dodecyl sulphate (SDS)
$C_{18}H_{29}NaO_3S$	Dodecyl benzenesulfonic acid (SDBS)
$C_{24}H_{39}NaO_5.xH_2O$	Sodium cholate or Na-cholate
Ca	Calcium
$CaCl_2$	Calcium chloride
CARS	Coherent anti-stokes raman scattering
CCAP	Culture collection of algae and protozoa
CeO_2	Cerium oxide

Chl	Chlorophyll
Chl-a	Chlorophyll-a
CAT	Catalase
cm	Centimetre
CM	Culture medium; algal assay procedure at U.S. Environmental Protection Agency
CNFs	Cellulose nanofibrils
CNT/s	Carbon Nanotube/s
Co	Cobalt
$CoCl_2$	Cobalt (II) chloride
Cr_2O_3 NMs	Chromium oxide (III) nanomaterials
Cu	Copper
$CuCl_2$	Copper (II) chloride
CuO	Copper oxide
DI	Deionized water
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
DWCNTs	Double Wall Carbon Nanotubes
EC_{50}	Half maximal effective concentration
ECHA	European chemicals agency
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
EPS	Exopolymeric substances
FCM	Flow cytometry
Fe	Iron

$FeCl_3$	Ferric chloride
FTIR	Fourier transform infrared spectroscopy
FU	Fluorescence units
g	Relative centrifugal force (or gram)
GA	Gum Arabic
GO	Graphene oxide
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
H_3BO_3	Boric acid
HD	Hydrodynamic Diameter
HLB	Hydrophyle–lyphophyle balance
HNO_3	Nitric acid
HWU	Heriot-Watt University
IC_{50}	Half maximal inhibition concentration
ICP-MS	Inductively coupled plasma mass spectrometry
IS	Ionic strength
ISO	International Standards Organisation
JRC	Joint Research Centre
KH_2PO_4	Monopotassium phosphate
L	Litre
LC_{50}	Half maximal lethal concentration
LM	Light microscope
Milli-Q water	Ultrapure water
mg/L	Milligram per litre

$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MHW	Moderately hard water
mL	Milliliter
mM	Millimolar
$MnCl_2$	Manganese(II) chloride
mV	Millivolt
MWCNT/s	Multi Wall Carbon Nanotube/s
Na	Sodium
Na_2MoO_4	Sodium molybdate
Na_2SiO_3	Sodium metasilicate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
ND	Not Determined
NH_4Cl	Ammonium chloride
Ni	Nickel
nm	Nanometre
NM	Nanomaterial
NMs	Nanomaterials
NM300k	Aqueous dispersion of Ag NM with stabilising agents from the JRC repository
NM300k DIS	The vehicle of the NM300k
NM400	Multi wall carbon nanotubes from the JRC Repository
NOEC	No Observed Effect Concentration
NOM	Natural organic matter
^oC	Degree celsius

OD	Optical density
OECD	Organisation for Economic Co-operation and Development
OPR	Oxygen production rate
Os	Osmium
Pb	Lead
PEC	Predicted environmental concentrations
pH	Potential Hydrogen
PPEI-EI	Poly(propionylethylenimine-co-ethylenimine)
PSII	Photosystem II (water-plastoquinone oxidoreductase)
PVP	Polyvinylpyrrolidone
QD	Quantum dots
ROS	Reactive oxygen species
RGB	Red, Green and Blue
rpm	Rotations per minute
s	Second
S	Sulphur
SAXS	Small-angle X-ray scattering
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard Error
SEM	Scanning electron microscope
Si	Silicon
SOD	Superoxide dismutase
SPG	Natural water samples from spring-fed river in north Florida, USA
SWCNTs	Single wall carbon nanotubes

TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
Triton X-15	Octylphenol Ethoxylate
µg/g	Microgram per gram
μm	Micrometre
µg/L	Microgram per litre
USEPA	United states environmental protection agency
UV	Ultraviolet
w/w	Weight concentration of a solution (weight per weight)
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffractometry
Zn	Zinc
$ZnCl_2$	Zinc chloride
ZnO	Zinc oxide
ZP	Zeta potential
ZVI	Zero-valent iron

Chapter 1

Introduction

Chapter 1 **Introduction**

1.1 Nanotechnology and nanomaterials

Nanotechnology is one of the fastest-growing technologies in the world (Blinova et al. 2010; Bondarenko et al. 2013). It is the study and application of very small particles called nanomaterials (NMs). The European Commission has defined a nanomaterial as: "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimension is in the size range 1–100 nm" (European Commission. 2011a).

NMs may be released into the environment from natural sources or through human activities. Natural sources include volcanic eruptions, natural dust, ocean spray, forest fires, soil erosion, weathering, clay minerals, and dust storms (Klaine et al. 2008b; Kumar et al. 2010a; Kabir et al. 2018). Human-related sources of NMs can be classed as intentional or accidental. The intentional can arise from the use of NMs in the treatment of contaminated ground water (for example zero-valent iron (ZVI) (Zhu et al. 2019), whereas accidental sources arise from combustion, for example zinc oxide (ZnO) (Khin et al. 2012). Industrial or manufactured NMs (as opposed to natural NMs) are used in a range of products or applications, including electronics, cosmetics, biomedicine and energy generation (Kumar et al. 2010b; Smita et al. 2012; Kabir et al. 2018). The accidental sources include vehicular emissions and mining/demolition (Peralta-Videa et al. 2011; Kabir et al. 2018).

After NMs are discharged into the environment, they might accumulate in different environmental media, such as water, air, soil, and sediments (Iavicoli et al. 2014). It is therefore important to assess the impact of NMs on the environment when assessing their safety.

1.2 Types of NMs

There are already several types of NMs under use and production, and new forms of NMs are expected to appear in the future (Kabir et al. 2018). Currently, NMs can be classified based on their composition (Rana and Kalaichelvan 2013; Saleh 2016) as follows:

- Carbon based (e.g. Fullerenes, Carbon nanotubes (CNTs))
- Metal based (e.g. Ag, Cu, Au, Fe, Co, Zn)

- Metal oxides (e.g. zinc oxide, iron oxide, titanium dioxide, cerium dioxide)
- Dendrimers (cascade molecules, quantum dots (Q-dots))
- Organic polymers (polystyrene, dendrimers, etc.).

These NMs are used in a wide range of products, such as cosmetics, health care, medical materials, clothing, toys, electronics and others (Thomas et al. 2006; Vance et al. 2015). Silver NMs (Ag NMs) (Wijnhoven et al. 2009) and carbon nanotubes (CNTs) (Mou et al. 2013), however, are the most commonly used NMs in consumer products; Ag NMs are mainly used for their antimicrobial properties (Wijnhoven et al. 2009) and CNTs for their unique mechanical and electrical properties (Mou et al. 2013).

1.3 NMs and the environment

NMs released into the environment can be interact with air, water and soil. This often changes the surface properties of the NMs which can result in agglomeration/aggregation or changes in particle charge and other surface properties (Handy et al. 2008a). The influence of changes of NM properties on the toxicity of NMs has been investigated in water ecosystems and soil, and can affect the fate and toxicity of NMs in the environment (Kiser et al. 2010; Quik et al. 2010). Therefore, understanding NM fate and behaviour needs to be assessed following their release into the environment in order to understand their environmental safety (Nowack 2009).

The environmental fate and behaviour of NMs are controlled by their physical, chemical and biological transformations (Lowry et al. 2012; Peijnenburg et al. 2015) as follows:

- Physical factors such as dispersion, agglomeration, formation, advection, deposition, replacement, or degradation of surface coating, deagglomeration and resuspension.
- Chemical factors, e.g. dissolution, aggregation, disaggregation and complexation with other chemicals, oxidation, and reduction reactions (redox), sorption, phase transformation and sulphidation. These may arise via interaction with organic matter.
- Biological factors such as phase transformations or degradation of the capping agent.

Aggregation and agglomeration are two of the key processes in aquatic systems, particularly affecting NM mobility, size and bioavailability (Baalousha et al. 2008,

2013). The International Organization for Standardization (ISO) (2015b) defines agglomeration as assemblages of particles held together by relatively weak forces (e.g. van der Waals, capillary, or electrostatic). In contrast, aggregation can be described as discrete assemblages of primary particles that are strongly bonded (i.e. fused, sintered, or metallically bonded) (ISO 2015b). In this thesis, the word agglomeration will be used to refer to both, when there is no clear distinction between these two phenomena. Text referring to published studies will use the authors' terminology.

The agglomeration of NMs has been shown to depend on particle properties (e.g. size, shape, surface roughness, surface charge, and concentration) and the physicochemical properties of the media (e.g. pH, ionic strength, counterion valency, and presence of organic macromolecules) (Bhattacharjee et al. 1998; Chen and Elimelech 2006; Chen et al. 2007; Baalousha et al. 2008; Baalousha et al. 2013). Regardless of the source, NMs can form aggregates (chemically bound) or agglomerates (physically bound) that may be considerably larger than the nanoscale (Handy et al. 2008b; ISO 2015a). Disaggregation and deagglomeration are important factors in determining the fate and behaviour of NMs in the natural environment (Newman and Stolzenbach 1996). Moreover, altering solution or media conditions such as ionic strength, pH, dilution and chemical composition might induce change in their particle aggregate/agglomerate structure or disaggregation/deagglomeration. For example, synthetic polymers have been shown to be able to separate aggregated NMs (Ouali and Pefferkorn 1994; Peijnenburg et al. 2015).

1.4 Nanotoxicology

Toxicology traditionally addresses adverse effects of chemicals to humans and environmental species (Elsaesser and Howard 2012). The most important parameters for conventional toxicology are concentration and time. As the size of materials decreases, their chemical reactivity typically increases (Jefferson 2000). Whilst there has been increasing research effort into investigating the toxicity of NMs to the environment, there are still gaps in knowledge that need to be addressed. Consequently, investigations into the toxicological potential of NMs have been continuously trying to catch up with the rapid growth of nanotechnology (Stone and Donaldson 2006). For nanotoxicology, NMs can be prepared in different ways for hazard studies (e.g. sonication, stirring, solvents, etc.), and this can influence their toxicity.

1.5 Algae and the environment

In most aquatic ecosystems, algae are the base of food webs, leading the biogeochemical cycling in the environment, and represent significant biodiversity (Wetzel 2001). Therefore, it is important to protect the natural levels of algal productivity in aquatic ecosystems to support food webs and biogeochemical cycling. Given the threat of regional and global change in environmental conditions, maintaining algal biodiversity is key to sustaining ecosystem function (Cardinale et al. 2006; Cardinale et al. 2012; Stevenson 2014).

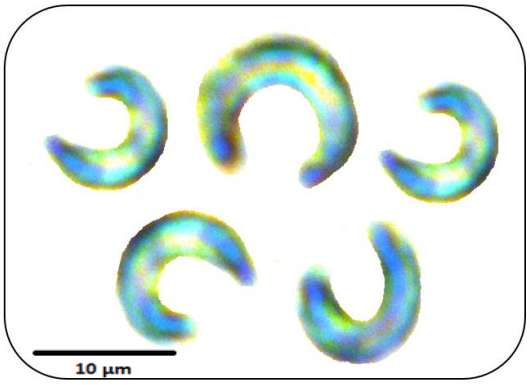


Figure 1.1: Green algae *Raphidocelis subcapitata*. Scale bar 10 µm.

Microalgae are used in the testing of chemicals for a variety of reasons, particularly the fact that they have well-defined and standardised test methods. One of the main species used, *Pseudokirchneriella subcapitata* (now *Raphidocelis subcapitata*), is a planktonic primary producer living in freshwater environments. Cells in culture are solitary except during cell division, occurring occasionally in confluence to form few-celled clusters enveloped by a delicate and colourless mucilage. Cells have a helical shape, usually semi-circularly curved in the vegetative phase, and become twisted in old cultures (Nygaard et al. 1986). The diameter of the 154–360 ° arc ranges from 4.8 to 10.8 µm, width from 1.6 to 4.4 µm and depth/width ratio from 1.7 to 4.1 (Figure 1.1). Microalga reproduction is by division of the mother cell into 2, 4 or 8 autospores (Nygaard et al. 1986); and (Guiry and guiry, 2019).

Investigation of the impact of NMs on algae can be used to assess the effects of NMs on the aquatic environment. In this research, the freshwater microalga *R. subcapitata* was used as a test organism to assess the effects of NMs.

1.6 Silver nanomaterials (Ag NMs)

Metal silver (Ag) is a naturally occurring and rare element, the sixty-seventh in abundance among the elements, and located forty-seventh in the periodic table (Liu

and Jiang 2015). As a precious metal and with a white, metallic, lustrous appearance, Ag has long been used as currency, jewellery, and high-quality cutlery (Liu and Jiang 2015). The use of Ag in medicine also has a long history. The first recorded medical use of Ag does date back to the eighth century (Liu and Jiang 2015).

The toxicity of Ag to humans is generally considered low (Tappin et al. 2010). Chronic exposure to Ag or Ag compounds may cause permanent grey or blue-grey discoloration of skin or eyes, e.g. argyris or argyrosis, systemic effects, immunological, neurological, developmental, reproductive and genotoxic effects, cancer and death (ATSDR 1990; Drake and Hazelwood 2005; Wadhera and Fung 2005). In terms of environmental effects, the ionic Ag is very toxic. However, it tends to bind rapidly with some compounds in the environment, such as compounds of sulphur and chloride, making it much less toxic (more details in section 1.6.2).

1.6.1 *Uses and applications of Ag NMs*

Silver nanomaterials (Ag NMs) are increasingly used in several applications, due to their unique physical and chemical properties such as industrial, household, and healthcare-related (e.g. medical device coatings, wound dressings) products and textiles, due to their antimicrobial properties, pharmaceuticals (e.g. drug delivery, anticancer agents) and in food storage, environmental, and biomedical applications (Sondi and Salopek-Sondi 2004; Li et al. 2010; Chernousova and Epple 2013; Li et al. 2014). A schematic diagram representing various applications of Ag NMs is provided in Figure 1.2.

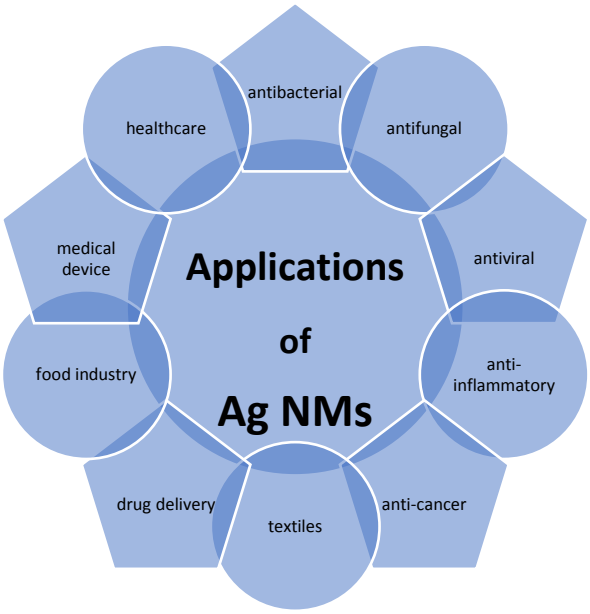


Figure 1.2: Various applications of Ag NMs.

These novel properties include optical, electrical, thermal, high electrically conductive properties, and also biological properties such as antimicrobial, antifungal, antiviral, anti-inflammatory, anti-cancer, and anti-angiogenic properties as a result of their surface-to-volume ratio (Li et al. 2001; Mukherjee et al. 2001; Sharma et al. 2009; Li et al. 2010; Gurunathan et al. 2015a; Peijnenburg et al. 2015; Zhang et al. 2016; Kumar et al. 2017). It has been suggested that such an increase in production and use of Ag NMs in many types of widely available products and applications might increase the presence of Ag NMs in the environment, particularly in aquatic systems.

The biological activity of Ag NMs depends on their physicochemical properties, including surface chemistry, size, size distribution, surface area, shape, composition, coating/capping, agglomeration/aggregation, and dissolution, and the type of reducing agents used for the synthesis of Ag NMs are a crucial factor for the determination of toxicity (Carlson et al. 2008).

1.6.2 *Ag release into the environment*

The main environmental exposure routes of Ag NMs found in textiles and cosmetics are through wastewater treatment plants and from industrial discharge. In most parts of the world, Ag released from wastewater treatment plants enters the aquatic environment through rivers and oceans (Peijnenburg et al. 2015). The Ag NM concentrations prevalent in ground and surface water exposed to Ag NMs is expected to be low. However, Ag released at concentrations toxic to some species is still possible (Hartemann et al. 2015). The predicted environmental concentrations (PEC) of Ag NMs in the EU have been proposed to be in the range of 30 to 80 ng/kg in natural and urban soils and from 1290 to 1390 ng/kg in sludge treated soils by 2020 (Layet et al. 2019). According to the United States Environmental Protection Agency (USEPA) (2003), the measurements of Ag in freshwater reservoirs show levels even below 0.01 μg/L in unpolluted areas and much lower concentration ranges were observed for Ag content in air (ng/m^3) (Strużyńska, 2019).

Physical processes such as agglomeration and sedimentation were found to play an important role controlling products containing Ag release in the natural surface water and therefore their fate in the environment (Petosa et al. 2010; Li and Lenhart 2012). Moreover, the dissolution of Ag NMs was found to be controlled by their concentration, surface coating, primary particle size and shape (Liu et al. 2010; Liu and Hurt 2010; Zhang et al. 2010a; Ma et al. 2011; Peretyazhko et al. 2014).

The release of Ag ions and the sulphidation process of Ag NMs are the most likely corrosion phenomena that may take place in the aquatic environment (Liu et al. 2010). Sulphidation can strongly affect surface properties of Ag NMs, including dissolution rate and surface charge, which will affect the toxicity and transport of Ag NMs in the environment (Levard et al. 2011). Moreover, sulphidation can make Ag NMs less toxic since it removes Ag ions from solution (Lowry et al. 2012). Furthermore, there are some environmental parameters that can influence NMs' behaviour. These parameters include pH, dissolved oxygen, ionic strength, ammonia content and chloride, salinity, dissolved organic carbon and temperature (Li et al. 2010; Liu et al. 2010; Zhang et al. 2010a; Kent and Vikesland 2012; Li and Lenhart 2012; Mumper et al. 2013; Peretyazhko et al. 2014).

1.6.3 *Fate and behaviour of Ag NMs in different environmental conditions*

The increased production, use and disposal of Ag NMs, and the development of personal care products containing Ag NM in recent years, may cause environmental Ag concentrations to increase (Mijnendonckx et al. 2013). Hartemann et al. (2015) highlight the wide use of Ag NMs and how environmental organisms are likely to be exposed to Ag NMs throughout the life of these Ag-NM-containing products. Therefore, distribution of Ag NMs in the environment, including the aquatic system, is likely to be widespread, and thus it is essential to assess the hazards that can be posed by these materials. Consequently, there is growing concern about Ag NMs' availability and increasing use in the environment, particularly their impact on aquatic systems. Knowledge of the measured/predicted concentrations of Ag NMs is important when designing hazard studies.

1.6.4 *Toxicity of Ag NMs to the aquatic environment*

Investigations into the toxicity of Ag NMs have previously reviewed their impact, and the impact of Ag salts, on diverse aquatic invertebrates, vertebrates, algae and bacteria (Peralta-Videa et al. 2011). A short-term study by Navarro et al. (2008b) reported the impact of $AgNO_3$ on growth inhibition of the algae *Chlamydomonas reinhardtii* and found that the IC_{50} values (median inhibition concentrations) were 31.9 and 31.2 µg/L after 1 and 2 h of exposure, respectively. In the same study, the authors investigated the impact of carbonate-coated Ag NMs on growth inhibition of the same microalga and found that the IC_{50} values were 356 µ/L after 1 h and 89 µg/L after 5 h of exposure.

This study therefore concluded that $AgNO_3$ toxicity was 11 times greater than Ag NMs based on IC_{50} values. This study looked at limited time points, and therefore consideration of toxicity over longer periods would be recommended to make comparisons between the toxicity of $AgNO_3$ and Ag NMs.

A study by Kennedy et al. (2010) investigated the acute toxicity of $AgNO_3$ and Ag NMs on three types of aquatic organism, including the alga *R. subcapitata*, for 48 h. They reported that the IC_{50} values for *R. subcapitata* were 9.9 and 20 µg/L for $AgNO_3$ and Ag NMs, respectively, suggesting that $AgNO_3$ was more toxic than Ag NMs. Kennedy et al. used a different medium, without EDTA, in their study (Bold's Basal Medium), rather than OECD 201 medium, and the pH was 8.06. Their results suggest that Ag NM suspensions were also significantly less acutely toxic than $AgNO_3$. Their findings indicate that the dissolved Ag^+ plays a critical role in acute toxicity of Ag NM to aquatic organisms. Therefore, the solubility of Ag NMs is likely to influence their toxicity.

Another study performed by Griffitt et al. (2008) on the effect of Cu, Ag, Ni and Co NMs in aquatic organisms – zebrafish (*Danio rerio*), adult *Daphnia pulex*, *Ceriodaphnia dubia* neonates, and algae (*R. subcapitata*) – found that the LC_{50} (median lethal concentration) for Ag NM coated with metal oxide (TiO_2) when exposed to *R. subcapitata* for 96 h was 190 µg/L, and when exposed to daphnids for 48 h it was 40 µg/L. These findings suggest that invertebrates (such as daphnids) and algae are more susceptible to toxicity from Ag NMs compared with zebrafish, since the LC_{50} (48 h) for zebrafish was 7.07 mg/L (Griffitt et al. 2008). Hence, this study illustrated that different species may vary in their sensitivity to NM toxicity. However, in order to confirm this, the toxicity of a wider selection of NMs would need to be assessed.

A study conducted by Ribeiro et al. (2014) investigated the toxicity of Ag NMs and $AgNO_3$ to *R. subcapitata*, *Daphnia magna* and *Danio rerio*. *D. magna* was the most sensitive organism and *D. rerio* was less sensitive than the other tested organisms. Results indicated that the IC_{50} for *R. subcapitata* exposed to $AgNO_3$ was 33.79 µg/L, and when exposed to Ag NM it was 32.40 µg/L, both after exposure for 72 h. The growth inhibition of *R. subcapitata* could be explained by the release of Ag^+ from Ag NMs. In this study a 24-well microplate was used for the exposure experiment, and the plate was shaken manually every 24 h to re-suspend any settled cells.

Gao et al. (2009) reported the impact of selected NMs (including Ag NMs) on *Ceriodaphnia dubia* following their dispersion in deionised water (DI water) and natural river water 48 h post using two different assay exposure conditions. The first assay assessed the toxicity of NMs to *Ceriodaphnia dubia*, and the second used the MetPLATE test as a bacterial enzyme toxicity test based on the inhibition of β-galactosidase. The NMs were less toxic when dispersed in river samples, suggesting that the dispersion method (in this case, medium) can influence NM toxicity. The *C. dubia* assay was much more sensitive, reporting higher toxicity of the NMs. This suggests that more than one approach should be used to evaluate NM toxicity.

McLaughlin and Bonzongo (2012) tested the effect of natural water chemistry on Ag NM behaviour and toxicity to the invertebrate *C. dubia* and the alga *Pseudokirchneriella* (now *Raphidocelis*) *subcapitata*. The results showed that the IC_{50} for *R. subcapitata* was 1,600 µg/L in the algal culture medium (CM), which prepared according to algal assay procedure at U.S. Environmental Protection Agency, and 22.6 ± 3.13 µg/L in the SPG water sample (natural water samples from a spring-fed river in northern Florida, USA). The higher the concentration of Ca^{2+} in the media, the greater the aggregation of NMs, and it was concluded that this influenced Ag NM toxicity. Furthermore, it was indicated that a small amount of organic matter (221 µg/L) in the SPG water protected *R. subcapitata* against Ag NM toxicity. Moreover, in terms of *C. dubia*, Ag NMs suspended in moderately hard water (MHW) and SPG water resulted in similar levels of toxicity response – 482 and 433 µg/L, respectively. Furthermore, the *C. dubia* was more sensitive to Ag NMs than *R. subcapitata*.

Książyk et al. (2015) examined the effect of Ag and platinum NMs to the freshwater microalga *R. subcapitata*, and found that the IC_{50} of Ag NMs was 1.6 mg/L. The authors used growth inhibition using an *in vitro* chlorophyll assay for 72 h.

It can be concluded from these studies that Ag NMs can exhibit toxicity to aquatic organisms. The IC_{50} values obtained for *R. subcapitata* exposed to Ag NMs ranged between 6.18 µg/L and 1.6 mg/L (Griffitt et al. 2008; Gao et al. 2009; Kennedy et al. 2010; McLaughlin and Bonzongo 2012; Ribeiro et al. 2014; Książyk et al. 2015; Curry 2017; Brown et al. 2018). The differences in toxic potency observed between experiments are likely to be due to NMs' physicochemical properties (e.g. dissolution, size, coating), media used, method of NM suspension, exposure period and of course endpoint studied.

Currently, NM concentrations used in many toxicology studies are much higher than what is expected in "real life" conditions in the environment (Fent et al. 2006; Muna et al. 2019). Time is another important factor that could impact the results, and thus it is important to consider exposure time when comparing results from different studies and evaluating their relevance.

Table 1.1 provides a summary of the biological effects of $AgNO_3$ and Ag NMs on microalgae and daphnids. The table includes details of the Ag NMs' physicochemical properties, NM dispersion protocols, different means of assessing impact on algae (e.g. cell counts, OD, Chl a), and the time points used. Information is also provided on daphnids for comparison purposes, given that they are the most studied species and thus important for comparison purposes. Daphnids are often considered the most sensitive species, and are certainly relevant when assessing the effects of NMs given their feeding mode (Lavorgna et al. 2016).

Based on the existing literature of the toxicity of Ag NMs to algae, the IC_{50} value were in the range of 20 – 1600 µg/L. These values were depending on experimental condition such as test species, time point, endpoint, temperature, light, shaker speed and NMs preparation. Moreover, the investigated endpoint was growth inhibition using a cell counter and *in vitro* chlorophyll a extraction. The tested time points were; 1, 2, 5, 24, 48, 72 and 96 h. It can be concluded that with increasing Ag NMs concentration, there were an increase in Ag NMs toxicity.

Table 1.1: A summary of the biological effects of $AgNO_3$ and Ag NMs on some microalgae and daphnia

Tested organism and NM preparation	Particle size and other physicochemical information	Endpoints investigated	Result and conclusion	Reference
Organism: *Chlamydomonas reinhardtii* **Exposure time:** 1–5 h **NM preparation:** Nanopure water was used, Ag NM freshly prepared before exposure. **Medium:** Major nutrients and EDTA trace metal solution.	Carbonate-coated Ag NM provided by NanoSys GmbH (Wolfhalden, Switzerland), 10 to 200 nm with most particles around 25 nm. Particles were investigated by researchers using TEM	Growth inhibition using a cell counter.	IC_{50} = 31.9 µg/L at 1h for $AgNO_3$ IC_{50} = 31.2 µg/L at 2 h for $AgNO_3$ IC_{50} = 356 µg/L at 1 h for Ag NM IC_{50} = 89 µg/L at 5 h for Ag NM Based on total Ag concentration, the toxicity of $AgNO_3$ was 11 times higher than Ag NM (in terms of IC_{50} values).	(Navarro et al. 2008b)
Organism: *R. subcapitata* **Exposure time:** 96 h **NM preparation:** 10 mg of Ag NM powder added to 10 mL of Milli-Q water, a 0.5% sodium citrate solution to help stabilise the suspensions, probe sonication for 6.5 seconds. **Medium:** Hard freshwater (dissolved oxygen, pH 8.2, hardness).	Ag NM coated with thin layers of metal oxide, Size 20–30 nm, provided by manufacturer. Ag NM was provided by Quantum Sphere (Santa Ana, CA, USA).	Growth inhibition assessed using in vitro chlorophyll a. Samples were incubated in a water bath at room temperature for 96 h under controlled light.	IC_{50} = 190 µg/L with algae IC_{50} = 7.07 mg/L with zebrafish The invertebrates (*daphnia*) and algae were more susceptible to toxicity from Ag NM compared with zebrafish.	(Griffitt et al. 2008)
Organism: *Ceriodaphnia dubia* **Exposure time:** 48 h **NM preparation:** Prepared in deionised water and filtered (0.45 µm) natural river water samples, sonication for 6.5 seconds. **Medium:** Natural river water samples.	20–30 nm metal-oxide-coated Ag NMs	Growth inhibition, 48 h *Ceriodaphnia dubia* assay and the MetPLATE test.	IC_{50} = 6.18 µg/L Regardless of the water matrix tested, Ag NM and Cu a NM were highly toxic to *C. dubia*. Water chemistry affects the suspension/solubility of NMs as well as the particle size distribution. Using DI water and drastic NM-suspension methods may not be as realistic as NM dispersion and suspension in natural waters.	(Gao et al. 2009)

Organism: *R. subcapitata* **Exposure time:** 96 h **NM preparation:** Addition of 75 mL of ethylene glycol, $AgNO_3$ was added before heating and stirring, water bath. **Medium:** Bold's Basal Medium without EDTA.	1) Ag NM dispersions (NC10, 20, 50 and 80 nm) 2) PVP Ag NM (polyvinylpyrrolidone)-coated Ag NMs, size 10–80 nm, obtained from NanoComposix (Biopure, San Diego, CA, USA).	Growth inhibition assessed using cell counts on a haemocytometer.	IC_{50} = 9.9 µg/L for $AgNO_3$ IC_{50} = 20 µg/L for two different sizes of Ag NMs Ag NM suspensions were significantly less acutely toxic than the disassociated Ag^+ ion. Dissolved Ag^+ plays a critical role in acute toxicity.	(Kennedy et al. 2010)
Organism: *R. subcapitata* **Concentration:** 0–100 µg/L **Exposure time:** 96 h **NM preparation:** Ag NMs were prepared by dispersing 200 mg Ag NM powder into 150 ml of water, then shaken for one week and then filtered at 1.6 µm. **Medium:** Growth culture medium (CM) shaken twice daily by hand.	Ag NM was 25.4 nm >99.9% purity of face-centered-cubic crystal structures, purchased from Quantum Sphere.	Growth inhibition assessed using in vitro chlorophyll a	IC_{50} = 1,600 µg/L in algal culture medium (CM) IC_{50} = 22.6 ± 3.13 µg/L in wetland water sample Higher concentration of Ca^{2+} causing greater aggregation. Small amount of organic matter in the SPG water was enough to protect against Ag NMs' toxicity to *R. subcapitata*. Organic matter plays a major role in the toxicity of Ag NMs.	(McLaughlin and Bonzongo 2012)
Organism: *R. subcapitata* **Concentration:** 0–125 µg/L **Exposure time:** 72 h in 24-well microplates. Every 24 h the plates were manually shaken, then read in a spectrophotometer at 440 nm. **NM preparation:** Dispersed in water. **Medium:** Woods Hole MBL culture medium.	$AgNO_3$ was purchased from Sigma-Aldrich as a crystalline powder, 99% purity. Ag NMs with an alkane coating, 3–8 nm size range, were supplied by AMEPOX (Poland).	Growth inhibition, 24-well microplates.	$AgNO_3$: IC_{50} was 33.79 µg/L (SE = 2.96) Ag NM: IC_{50} was 32.40 µg/L (SE = 2.09) The toxicity scenario inverts: at 25µg/L, Ag NM is twice as toxic as $AgNO_3$, whereas at 50 µg/L, Ag NM is nearly half as toxic as $AgNO_3$, which causes 100% growth inhibition. This could be explained by the continuous release of Ag^+. Toxicity is highly dependent on the chemical composition of the medium.	(Ribeiro et al. 2014)
Organism: *R. subcapitata* **Concentration:** 1, 2, 3, 4 and 5 mg/L **Exposure time:** 72 h **NM preparation:** Used Milli-Q water sonicated for 30 min. **Medium:** Algaltoxkit FTM, activated culture, the initial density of cells was 10^6/mL	Ag NM nanopowder 34 ± 18 nm non-coated, purchased from Sigma-Aldrich, Poland.	Growth inhibition assessed using in vitro chlorophyll a.	IC_{50} = 1.6 mg/L Ag NMs were highly toxic to *R. subcapitata* even at very low concentrations. The result was 8 times lower than the result obtained by Griffitt et al. (2009). The difference could be related to the particle size.	(Książyk et al. 2015)

Organism: *R. subcapitata* **Concentration:** 0, 0.2, 0.6, 2, 6, 20 µg/L **Exposure time:** 72 h **NM preparation:** Sonicated twice for 8 min. **Medium:** OECD 201	Ag NMs <20 nm, provided by the manufacturer.	Growth inhibition (OECD 201).	IC_{50} = 7.37 µg/L as Ag for $AgNO_3$ IC_{50} = 66–173 µg/L for Ag NMs IC_{50} based on OECD medium.	(Curry 2017)
Organism: *R. subcapitata* **Concentration:** 0.001–5 mg/L **Exposure time:** 72 h **NM preparation:** Sonicated for 16 min. **Medium:** OECD 201	Ag NMs 10 nm provided by the manufacturer, Plasmachem.	Growth inhibition (OECD 201).	IC_{50} = 0.1 mg/l Ag NMs more toxic than ZnO (2.5 mg/L) and QDs (3.5 mg/L).	(Brown et al. 2018)

1.7 Carbon nanotubes (CNTs)

There are several different types of carbon, based on bonding relationships with the neighbouring carbon atoms. Carbonaceous NMs share the same bonding configurations as macroscopic carbon structures. Mauter and Elimelech (2008) described various morphological structures of carbon-based NMs such as nanodiamond, fullerene (C60), fullerene (C540), carbon onion, single-walled nanotubes (SWCNT), multi-walled nanotubes (MWCNT) and graphene (Figure 1.3). The physical, chemical, and electronic properties of CNTs are strongly coupled to their structural conformation (Ajayan 1999).

Figure 1.3: Carbon-based nanomaterials. Many chemical and electronic properties of carbonaceous nanomaterials are determined by the dominant hybridisation state of the carbon-carbon bonds. Source: (Mauter and Elimelech 2008).

There are 3 different standard types of CNTs. Firstly, single-walled carbon nanotubes (SWCNTs) consist of a single graphite sheet wrapped into a cylindrical tube. Secondly, double-walled carbon nanotubes (DWCNTs) consist of two layers of carbon walls. Thirdly, multi-walled carbon nanotubes (MWCNTs), which are comprised of multiple layers of single-walled carbon nanotubes (Jackson et al. 2013) (Figure 1.4). CNT source materials are usually self-entangled and agglomerated. The CNTs in these buckypapers tend to maintain their straight morphology (Wang et al. 2008). CNTs are unique "one-dimensional" fibres, and their diameter typically varies in the range 0.4–40 nm. However, the length can vary from 0.14 nm to 5.5cm (Zhang et al. 2013).

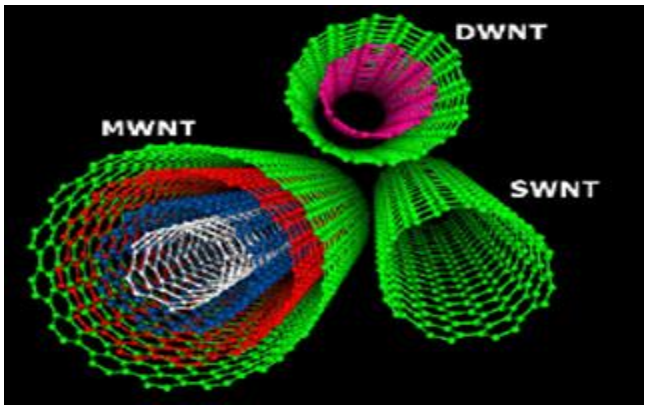


Figure 1.4: Single-walled, double-walled and multi-walled carbon nanotube structures (Source: GIST LMNF 2015).

1.7.1 *Uses and applications of CNTs*

CNTs have unique properties, such as extraordinary strength and the ability to transfer heat in a very efficient way, and electronically they can be semiconductors, making them potentially useful in many applications (Figure 1.5). Products and applications that exploit CNTs include electronics, energy storage and production, and biomedical applications (such as drug delivery systems and scaffolds) (Baughman et al. 2002).

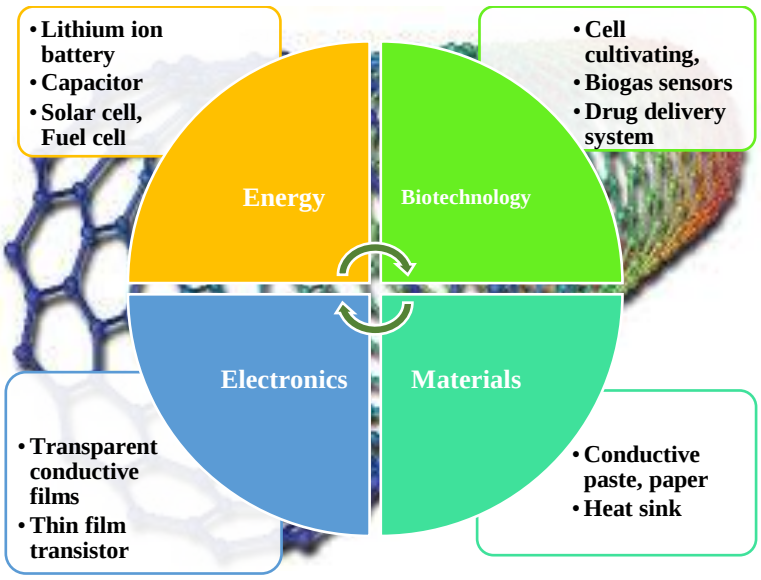


Figure 1.5: Some applications of carbon nanotubes.

1.7.2 *CNT release into the environment*

CNTs are likely to be released into the environment given the increase in their production and their use in a wide range of industrial and consumer products (Lukhele et al. 2015). The predicted environmental concentrations (PEC) of CNTs have been proposed to be in the range of 0.001–1000 µg/L (De Marchi et al. 2018; Yan et al. 2019). Despite the different environmental hazard studies using CNTs, it is still not clear what the toxicity pathways and possible toxic effects of CNTs in the environment might be (Mueller and Nowack 2008; Gottschalk et al. 2009).

1.7.3 *Fate and behaviour of MWCNTs in different environmental conditions*

MWCNTs can be released to the environment during the life cycle of products containing MWCNTs, or through their combustion. Subsequently, environmental parameters will affect and condition the fate and behaviour of the CNTs (Peijnenburg et al. 2015). In aquatic environments, CNTs can be removed from the water column

by agglomeration with natural organic matter (NOM) and sedimentation (Schwyzer et al. 2012).

Moreover, the degree of agglomeration is based on aromaticity and charge density of NOM (Hyung and Kim 2008; Chappell et al. 2009). Furthermore, CNTs' properties, such as length, diameter and functionalisation, have an influence on the interaction between CNTs and NOM in aquatic environments (Hyung et al. 2007; O'Driscoll et al. 2010; Schwyzer et al. 2012).

The receiving medium has an effect on agglomeration and sedimentation of the CNTs in aquatic media (Koelmans et al. 2009; Chen et al. 2010; Praetorius et al. 2012). For instance, the ionic strength, pH, concentration and type of the NOM, as well as the valence and the concentration of cation in the media, can affect the stability, fate and behaviour of CNTs in the aquatic media (Hyung et al. 2007; Domingos et al. 2009; Chen et al. 2012a; Gigault et al. 2012).

The ionic strength of the medium is an important factor affecting the stability of CNTs in suspension (Schwyzer et al. 2012). With increasing ionic strength the stability effect of adsorbed NOM can be reduced, leading to higher agglomeration and then sedimentation of the CNTs (Hyung and Kim 2008; Yang and Xing 2010). In terms of the pH of the media, NOM adsorption to NMs is affected by the pH. At higher pH values, there will be a higher electrostatic repulsion and fewer hydrophobic and hydrogen-bond interactions, which likely lead to lower NOM adsorption (Hyung and Kim 2008; Yang and Xing 2010).

1.7.4 *Toxicity of MWCNTs and CNTs to aquatic environment*

Several studies have investigated the toxicity of NMs in the aquatic environment using algae as a test species. A summary of their results is provided below and in Table 1.2. Investigations of the toxicity of CNTs have previously included their impact on diverse aquatic organisms (reviewed in Jackson et al. 2013). These authors found that pristine or functionalised CNTs have different dispersion behaviour, potentially leading to different risks of exposure along the aquatic environment. Moreover, CNTs do not cross biological barriers readily (Jackson et al. 2013). Furthermore, CNT toxicity depends on exposure conditions, CNT type, model organism, concentration and dispersion state. The authors conclude that SWCNTs were found to be more toxic than (double-walled carbon nanotubes) DWCNTs and MWCNTs (Jackson et al. 2013).

A study by Schwab et al. (2011) investigated the effect of agglomerated and well-dispersed carbon nanotubes on two species of algae (*Chlorella vulgaris* and *R. subcapitata*). In their study, two different types of MWCNTs were used: pristine and oxidised. NOM was incorporated into the algal medium to prepare a "well-dispersed" MWCNT suspension. All preparations of MWCNTs were bath sonicated prior to use. The IC_{50} values were 24 and 1.8 mg MWCNT/L for *C. vulgaris* after 72 h of exposure to agglomerated and well-dispersed MWCNTs, respectively. On the other hand, the IC_{50} values were 36 and 20 mg MWCNT/L for *R. subcapitata* when exposed to agglomerated and well-dispersed MWCNTs, respectively. *R. subcapitata* was therefore found to be less sensitive to MWCNTs than *C. vulgaris*, and well-dispersed MWCNTs were more toxic than agglomerated samples. The interaction of algae with CNTs was investigated using bright field and fluorescence microscopy. The majority of algal cells were located within MWCNT agglomerates. Cells of *C. vulgaris* were located more often in MWCNT agglomerates than cells of *R. subcapitata*. Moreover, the authors indicate that there is interaction between CNTs and the algal surface.

In research conducted by Gao et al. (2012), the influence of the dispersion method on the toxicity of carbon-based nanomaterials was tested on model aquatic organisms. The authors selected gum arabic (GA) (10 g/L) as a nontoxic surfactant and used it to prepare suspensions of C_{60} and SWCNTs. The suspensions were homogenised using a high shear IKA T-25 Ultra-Turrax mixer for approximately 1.5 h, followed by ultrasonic bath sonication using a Misonix S3000 for 10 min. The mixtures were then ultracentrifuged at 44,000 g for 2.5 h. After this, the supernatant was separated and impurities removed from the agglomerated NMs at the bottom of the tubes. After 96 h, the growth inhibition of *R. subcapitata* was determined by measuring the concentration of chlorophyll a (Chl a). Different types of surfactant were used based on the different preparation methods of NM suspensions, these surfactants were; Poly(vinylpyrrolidone) (PVP) ($[C_6H_9NO]_n$), tetrahydrofuran (THF) (C_4H_8O), GA, SDBS ($C_{18}H_{29}NaO_3S$), SDS ($C_{12}H_{25}O_4SNa$), sodium cholate or Na-cholate ($C_{24}H_{39}NaO_5.xH_2O$), Triton X-15 (or glycols, polyethylene, mono [(1,1,3,3-tetramethylbutyl) phenyl] ether), and Triton X-100 ($[C_2H_4O]_nC_{14}H_{22}O$).

The results showed that the IC_{50} values were 0.27 mg/L at 48 h for *Ceriodaphnia dubia* (the end point was mortality), and 2.11 mg/L at 96 h for *R. subcapitata* (the end point was growth inhibition). The results also showed that Na-cholate, Triton X-15, PVP and GA had no significant negative biological impacts on *R. subcapitata*. In contrast,

only PVP and GA showed similar effects on *C. dubia*. SDS had the highest toxic effect, with an IC_{50} of 3 mg/L for the *C. dubia* bioassay, but an IC_{50} of only 73.8 mg/L when *R. subcapitata* was used. For SDBS, an IC_{50} of 10 mg/L was obtained with the *C. dubia* 48-h acute toxicity assay and an IC_{50} of 79.3 mg/L was observed with *R. subcapitata*. These findings suggest that purity and degree of agglomeration of SWCNTs, as well as the type of surfactant used to help with the dispersion process, were responsible for toxicity to tested organisms. Furthermore, *C. dubia* was more sensitive than *R. subcapitata*.

Pereira et al. (2014) evaluated whether MWCNTs and cellulose nanofibers (CNFs) were toxic to *C. vulgaris* in Bold's Basal Medium (BBM) or in natural fresh water (from the River Seine). In their study, the impact of NMs on algae membrane integrity was assessed using the Trypan Blue assay. MWCNTs in the BBM caused a significant reduction of cell viability at all concentrations tested (45.50–69.83% relative to controls). However, for cellulose CNFs (1 and 50 μg/mL), the toxicity (50.50 and 48.83%,) was only observed over 72 h of exposure. This suggests that MWCNTs were more toxic than CNFs. Cells incubated in Seine river water during exposure to MWCNTs did not show a decrease in viability from 1 μg/mL to 72 h, however, at 96 h, a reduction in cell viability to 61.70% (p=0.038) was observed, as compared to the control. Moreover, MWCNTs were less toxic when dispersed in Seine river water compared to BBM. For CNFs in the Seine river water, all tested concentrations were toxic. Furthermore, both NMs had an effect on algal growth and caused cell death. In addition, cellular uptake of MWCNTs was observed in algal cells cultured in BB culture medium; however cells cultured in Seine river water were not seen to internalise MWCNTs.

Lukhele et al. (2015) evaluated the acute toxicity of DWCNTs to 3 aquatic organisms (*R. subcapitata*, *Daphnia pulex* and *Poecilia reticulata*). DWCNT suspensions were prepared in media of varied ionic strength and humic acid concentrations. The suspensions were bath sonicated for 2 h and shaken at 30-min intervals. The suspensions were refrigerated at 4 °C for 24 h before being used (to stabilise the suspensions). Increasing ionic strength (IS) caused an increased agglomeration of both pristine and oxidised DWCNTs. The addition of sodium chloride to the DWCNT suspensions resulted in increased agglomeration in both pristine and oxidised DWCNTs as the IS increased.

Moreover, the growth inhibition caused by oxidised DWCNTs was lower than that of pristine DWCNTs. The IC_{50} of pristine DWCNTs was found to be 17.95 mg/L, while oxidised DWCNT exposure resulted in an IC_{50} of 10.93 mg/L. In this study, the authors observed the clustering of *R. subcapitata* within DWCNT agglomerates using a light microscope. The study concluded that humic acid content and ionic strength resulted in a reduction of toxicity of both pristine and oxidised DWCNTs to *R. subcapitata*.

Sohn et al. (2015) investigated the acute toxicity of SWCNTs towards two freshwater microalgae (*R. subcapitata* and *C. vulgaris*), a microcrustacean (*D. magna*), and a fish (*Oryzias latipes*) based on OECD test guidelines (201, 202, and 203, respectively). SWCNTs had a tangled shape and were ~20 μm in length and ~1-1.2 nm in diameter. The SWCNTs were dispersed in distilled water containing 0.01% bovine serum albumin (BSA), and sonicated six times for 30 min in an ultrasonic bath sonicator. BSA was chosen to prepare the suspensions of SWCNTs and maintain the stability of the dispersion phase for a long period. Distilled water and vehicle (BSA) controls were both used in all the toxicity tests, and the BSA control was not found to induce any adverse effects compared to the control sample (without BSA). Based on the average specific growth rate, the results showed that IC_{50} values were not significantly different to each other; they were 29.99 and 30.96 mg/L for *R. subcapitata* and *C. vulgaris*, respectively. In contrast, the SWCNTs did not cause any acute toxicity against the *D. magna* and freshwater fish *O. latipes* up to a concentration of 100 mg/L. The authors concluded that the major contributor at a lower concentration and smaller size was oxidative stress, whereas the contribution of shading and agglomeration increased with concentration. Furthermore, SWCNTs could induce acute toxicity in freshwater algae (*R. subcapitata* and *C. vulgaris*), yet not in *D. magna* and *O. latipes*. Consequently, SWCNTs would seem to have different short-term effects on different species of aquatic organisms.

Rhiem et al. (2015) reported on the interactions of MWCNTs with algal cells. The CNTs were dispersed in the algal medium (OECD 201) by means of probe ultrasonication for 5 min. The sonication procedure was repeated 4 times or more until no more agglomerated CNT material was macroscopically visible. This method resulted in the presence of small agglomerates and single tubes of 0.2–1.0 mm mean tube length (both referred to as CNTs) in the medium, as was visualised by TEM. The MWCNTs interacted with *Desmodesmus subspicatus* cells and were found attached

to the outer cell wall and the inner cell membrane, and lying in the cytoplasm at exposed algae compared to control sample. Algae were exposed to ^{14}C radiolabelled CNTs, and quantification of CNT accumulation was based on separation of cells and nanomaterial by density gradient centrifugation with a colloidal silica water gradient. Transfer of CNT-exposed algae to uncontaminated medium led to dissociation of the CNT material, resulting in a reduction of the algae CNT concentration by 80% within 3 days.

Table 1.2: A summary of the biological effects of CNTs on microalgae

Tested organism and CNTs used	Type of CNT	Size and length	Dispersion method	Sonication time	Endpoints investigated	IC_{50} values and result	Reference
Organism: Alga *Thalassiosira pseudonana*, copepod *Tigriopus japonicus* and medaka *Oryzias melastigma* **Exposure time:** 96 h **Stock concentration:** 100 mg/L **Concentration:** 0.1–100 mg/L **Medium:** f/2-Si medium "artificial seawater" ASW	DWCNT	0.7–2.1 nm Length unknown	Dispersed DWCNTs in ASW using: 1: ultrasonic bath for 2 h 2: continuous stirring with a magnetic stirrer (~200 rpm) for 2 weeks.	2 h and 2 weeks	Growth inhibition based on cell counting using a haemocytometer	IC_{50} for the alga was 1.86 mg/L using bath sonication and 22.7 mg/L using continuous stirring. Sonication resulted in better dispersal of DWCNTs in artificial seawater than stirring.	(Kwok et al. 2010)
Organism: *R. subcapitata* and *C. vulgaris* **Exposure time: 96 h** **Stock concentration:** Different **Concentration**: 0.1–100 mg/L **Medium:** OECD	Pristine and oxidised CNT	1–1,000 nm	50.0 mg of CNT and 15 mL of 400 mg NOM/L were sonicated in 100 mL flasks (2 × 15 min) in an ultrasonication bath.	2 × 15 min	OECD 201 guideline combined with photosynthetic activity measurements	IC_{50} was 1.8 mg/L in well-dispersed CNTs and 24 mg/L in agglomerated suspensions of CNTs. Growth inhibition was highly correlated with the shading of CNTs and the agglomeration of algal cells.	(Schwab et al. 2011)
Organism: *R. subcapitata* and *Ceriodaphnia dubia* **Exposure time:** 96 h for *R. subcapitata*, 48 h for *C. dubia* **Stock concentration:** 200 mg/L **Concentration:** ND **Medium:** USEPA	C_{60} and SWCNT	35.8 (±1.7) nm and 1.1 nm with avg. length 375 (±75) nm	NM mixed with gum arabic (GA) then homogenised using a high-shear IKA T-25 Ultra-Turrax mixer for 1.5 h followed by ultrasonication.	10 min	Growth inhibition using chlorophyll a (Chl-a).	IC_{50} value (96 h) for C_{60} with GA was 0.139 mg/L and IC_{50} value for SWCNT with GA was 2.11 mg/L. Toxicity of SWCNTs was dependent on purity and degree of aggregation. The type of surfactant used to help with the dispersion process in aqueous solutions should not be overlooked.	(Gao et al. 2012)

Organism: *Chlorella sp.* **Exposure time:** 96 h **Stock concentration:** 100 mg/L **Concentration:** 0, 5, 10, 20, 50, and 100 mg/L **Medium:** OECD medium	MWCNT -CNT10 -CNT40 -CNT100	<10 nm 20–40 nm 60–100 nm	MWCNTs and AC (100 mg/L) were dispersed in the culture medium using bath sonication.	15 min	Growth inhibition using a counting chamber under a light microscope.	MWCNTs significantly inhibited algal growth. The 96-h IC_{50} values were 41.0, 12.7, and 12.4 mg/L. Lower concentration and small size led to oxidative stress. Shading and agglomeration increased with increased MWCNT concentration.	(Long et al. 2012)
Organism: *Scenedesmus obliquus* **Exposure time:** 96 h **Stock concentration:** 1,000 mg/L **Concentration:** 0.1–100 mg/L for SWCNT & 0.1–200 mg/L for DWCNT **Medium:** M11	SWCNT Short SWCNT DWCNT Short DWCNT	1–2 nm 1–2 nm 2–4 nm 2–4 nm	Dispersed in aqueous HNO_3 (2.6M). Remained for 48 h. Centrifuged for 10 min. Washed with deionised water. Re-suspended in deionised water by ultrasonication.	ND	OECD 201 growth inhibition based on cell counting using a haemocytometer	33.88 mg/L for 72 h. 37.58 mg/L for 72 h. 45.79 mg/L for 72 h. 65.06 mg/L for 72 h.	(Mou et al. 2013)
Organism: *Chlorella vulgaris* **Exposure time:** 24, 48, 72 and 96 h **Stock concentration:** 10 mg/mL **Concentration:** 1–100 mg/L **Medium:** BB culture and Seine river water	MWCNT and CNF	20–40 nm and lengths of 40–60 µm	MWCNTs washed and filtered several times. Dried at 80 °C for 12 h. Sonicated in ice bath.	5 min for BB culture and 20 min for Seine river water	Growth inhibition based on cell counting.	MWCNTs and CNFs affect cell viability and algal growth.	(Pereira et al. 2014)
Organism: *R. subcapitata*, *Daphnia pulex* and *Poecilia reticulata* **Exposure time:** 24, 48 and 72 h **Stock concentration:** 100 mg/L **Concentration:** ND **Medium:** Algaltoxkit OECD	Pristine and oxidised DWCNTs	3.6 nm	10 mg of DWCNT in 100 mL of culture medium. Bath ultrasonication. The suspensions were prepared 24 h before toxicity tests were carried out, and were refrigerated at 4 °C.	2 h	Growth inhibition using a spectrophotometer and confirmed using manual cell counting.	The toxicity of DWCNTs to 3 different species varied. *D. pulex* was the most sensitive organism, followed by alga and fish. Humic acid increased the toxicity of DWCNTs to *D. pulex* and *P. reticulata*, while increased ionic strength reduced the toxicity of DWCNTs to these organisms. Both humic acid and increased ionic strength reduced the toxicity of DWCNTs in *R. subcapitata*.	(Lukhele et al. 2015)

Organism: *R. subcapitata* and *C. vulgaris* **Exposure time:** 72 h **Stock concentration:** ND **Concentration:** 12–46.10 mg/L **Medium:** OECD	SWCNT	1–1.2 nm and ~20 μm in length	0.5% bovine serum albumin (BSA).	ND	Growth inhibition based on cell counting using a haemocytometer	IC_{50} was 29.99 mg/L for *R. subcapitata* and 30.96 mg/L for C. *vulgaris*. Shading was the major cause of growth inhibition in the freshwater microalga.	(Sohn et al. 2015)
Organism: *Desmodesmus subspicatus* **Exposure time:** 24, 48 and 72 h **Stock concentration:** 2 mg/L **Concentration:** 1 mg/L **Medium:** OECD	MWCNT	4 nm inner and 5–20 nm outer diameter	MWCNTs dispersed in water by ultrasonication (0.2-second pulse at 70 W followed by 0.8-second pause) for 5 min. repeated 4 times.	5 min × 4	Visualisation of CNT uptake in cells. Effects of CNTs on algal cell biochemistry	CNTs did not influence algal growth during the accumulation phase. After transfer of CNT-exposed algae to clear water, a time-dependent release of CNT material from the cells was observed.	(Rhiem et al. 2015)
Organism*:* *Chlorella pyrenoidosa* **Exposure time:** 96 h **Stock concentration:** ND **Concentration:** 1–100 mg/L **Medium:** OECD medium	MWCNT	60–100 nm	DOM (5 mg/L) were bath sonicated. All of the media, with and without the test materials, were autoclaved at 0.1 MPa for 20 min and bath sonicated for 20 min.	20 min	Growth inhibition using a counting chamber under a light microscope.	MWCNTs could significantly inhibit algal growth through elevated oxidative stress, physical interaction with cells, and the shading effect. The 3 DOMs had largely comparable capabilities of dispersing and stabilising MWCNTs in the OECD medium and inhibiting the hetero-agglomeration and co-precipitation of MWCNTs with algal cells. IC_{50} was 14.5 ± 1.1 without DOM. MWCNTs + HA = 23.3 ± 1.6 MWCNTs + SDBS = 10.0 ± 1.7 MWCNTs + TX100 = 12.6 ± 0.6	(Zhang et al. 2014)

It can be concluded from these studies that the IC_{50} values obtained for *R. subcapitata* exposed to MWCNTs ranged between 1.8 mg/L and 41 mg/L (Schwab et al. 2011; Long et al. 2012; Pereira et al. 2014; Zhang et al. 2014; Rhiem et al. 2015). Generally, the effect of CNTs depends on several factors: type of species; degree of agglomeration; dissolution of impurities; NM size and length; coating; media, and method of suspension, as well as endpoint studied. The exposure to MWCNTs may cause effects to aquatic organisms, in particular *R. subcapitata*. Based on the IC_{50} value (inhibition of growth), the CNTs have a toxicity as high as 1.8 mg/L in well-dispersed CNTs in the presence of NOM, and 24 mg/L in agglomerated suspensions of CNT without NOM. Moreover, the investigated endpoint were growth inhibition and photosynthetic activity using a cell counter and *in vitro* chlorophyll a extraction. The tested time points were; 24, 48, 72 and 96 h. It can be concluded that with well-dispersed CNTs, there were an increase in CNTs toxicity compared to agglomerated suspensions of CNTs.

1.8 Factors affecting of NMs toxicity

There are some factors affecting NMs toxicity to inhibit algal growth, such as metal catalyst residues, solubility, oxidative stress, shading effects and the adsorption of nutrient elements.

1.8.1 *Metal trace elements*

CNTs often contain residual metal impurities such as Fe, Ni, Co and Mo, even after prolonged periods of purifying with concentrated nitric acid at a temperature of 80 °C (Pumera 2007; Bennett et al. 2013). The main source of impurities in CNTs is their synthesis method. CNTs are usually produced by chemical vapour deposition or arch-evaporation synthesis where metal catalyst nanomaterials are used (mostly nickel, cobalt or iron) (See and Harris 2007). The presence of metal impurities can enhance CNT toxicity (Choi et al. 2006; Pulskamp et al. 2007; Porter et al. 2009; Tan et al. 2009; Basiuk et al. 2011). However, it has also been reported that NMs with low iron content induce toxic effects toward mice (Shvedova et al. 2005).

Furthermore, no induced algal toxicity of the leachates from the pristine MWCNTs was observed in a study by Long et al. (2012), and evidence suggested that catalyst residues were not responsible for the toxicity of the MWCNTs to *Chlorella sp*. The toxic mechanisms of pristine MWCNTs likely resulted from 3 main factors: oxidative

stress; agglomeration and physical interactions with the cells, and shading effects (Long et al. 2012).

Shvedova et al. (2012) indicate that the major source of oxidative stress is transition metal-based NMs or transition metal contaminants used as catalysts during the production of non-metal NMs, such as SWCNTs and MWCNTs. Furthermore, Saxena (2018) reported that the impurities of NMs by metal residue are presumed to be involved in imparting toxicity to algae.

1.8.2 *Dissolution*

Dissolution is a critical environmental transformation process that determines the ultimate effects of Ag NMs in the environment and within organisms. Liu et al. (2010) and Liu and Hurt (2010) indicated that Ag^+ release from Ag NMs is a cooperative oxidation process requiring both protons (H^+) and the dissolved O_2. Moreover, the adsorption of natural organic matter (NOM) to NM surfaces prevents oxidative dissolution (Fabrega et al. 2009; Yang et al. 2014). Ma et al. (2011) indicate that the dissolution of Ag NMs can affect their toxicity and persistence in the environment. Moreover, differences in experimental conditions, such as pH and dissolved oxygen concentration, make it difficult to identify which NM and solution properties control the dissolution of Ag NMs (Ma et al. 2011).

1.8.3 *Oxidative stress*

The term "oxidative stress" refers to an imbalance between reactive oxygen species (ROS) production and antioxidant defences (Johnston et al. 2018). Sies (1985) defined this as "a disturbance in the prooxidant–antioxidant balance in favour of the former, leading to potential damage". An abundance of ROS can potentially lead to damage to biological molecules (e.g. proteins, lipids, DNA), resulting in oxidative stress and cell damage. To minimise the excess ROS response, cells have enzymatic and nonenzymatic antioxidants which defend against ROS (Ahmad et al. 2010; Maya and Matsubara 2013).

The link between NM toxicity and oxidative stress has been widely reported, particularly when investigating impacts on human health (Nel et al. 2006). Moreover, some studies indicated that oxidative damage was involved in the toxicity of Ag NMs to animals, bacteria, and algae (Oukarroum et al. 2013; Jiang et al. 2014).

Ag NMs can induce oxidative stress in *Wolffia globosa* by blocking photosynthetic electron transfer (Zou et al. 2016). Modelling of the relative positions showed that a considerable number of oxide NMs (e.g. CuO, TiO_2, FeO, Fe_2O_3 and ZnO, etc.) are capable of unbalancing the cellular redox state by redox interactions, and therefore are potentially also capable of causing oxidative stress in aquatic environments (Moos and Slaveykova 2014).

In terms of CNTs, there is evidence that exposure to CNTs can induce oxidative stress in algal cells, macrophages, human keratinocytes and rat aortic endothelial cells (Cheng et al. 2001; Pulskamp et al. 2007; Gaiser et al. 2011; Di Giorgio et al. 2011; Pereira et al. 2014). Similarly, catalyst metal residues can lead to oxidative stress (Saxena 2018). In contrast, Schwab et al. (2011) concluded that the effects of CNTs on *R. subcapitata* could not be attributed to dissolved and insoluble metal impurities of CNTs. They stated that metal impurities potentially associated with CNTs were very unlikely to be the cause of the CNTs' toxic effects, as they are not the main factor in CNT toxicity (Schwab et al. 2011). In general, it can be concluded that the oxidative stress of NMs is one of the most important factor for NMs toxicity and have an impact on living organism cussing cell damage.

1.8.4 *Shading effect and cell agglomeration with NMs*

These physical interactions have a remarkable impact in the observed toxic effects. Some studies revealed the influence of physical interactions of NMs with algal cells such as SiO_2 NMs with *R. subcapitata* (Hoecke et al. 2008), Cu NMs with *Microcystis aeruginosa* (Wang et al. 2011) and CeO_2 NMs with *Chlamydomonas reinhardtii* (Röhder et al. 2014).

With regards to CNTs, a shading effect is very often considered to explain their toxicity through reduced availability of light to phototrophic organisms (Hoecke et al. 2008). Schwab et al. (2011) indicated shading and agglomeration of CNTs as the reasons for their toxicity to algae. These authors indicated that 85% of the total toxicity was caused by the shading effect of CNTs and the agglomeration of microalgal cells with CNTs (Schwab et al. 2011). These authors found that up to 95% of all algal cells were agglomerated with CNTs after 96 h (*C. vulgaris* and oxidised CNTs, 50 mg CNT/L). The lowest CNT concentration that caused significant agglomeration was 1.3 mg well-dispersed CNT/L (*C. vulgaris* + CNTs). Well dispersed CNTs (with NOM) agglomerated with algal cells almost ten times more than agglomerated CNT (without

NOM) suspensions. For instance, 50 mg agglomerated CNT/L and 5.5 mg well-dispersed CNT/L led to a comparable agglomeration for *C. vulgaris*. *R. subcapitata* cells agglomerated with CNT were 77% less compared to agglomerated *C. vulgaris* cells with CNTs.

1.8.5 *The adsorption of nutrient elements*

One of the toxicity mechanisms of NMs is adsorption of nutrient elements from the surrounding environment due to NMs' high surface area. TiO_2 NMs can adsorb Zn and P from algal growth medium and thus limit the availability of these nutrients to algae (Kuwabara et al. 1986). Toxicity differences between NMs could be caused by depletion of phosphate reaching the algal cell surface due to adsorption to the particle surfaces (Rogers et al. 2010).

Hoecke et al. (2009) investigated the fate and effects of CeO_2 NMs in aquatic ecotoxicity tests using 4 different organisms – the green alga *R. subcapitata*, the crustaceans *Daphnia magna* and *Thamnocephalus platyurus*, and embryos of *Danio rerio*. Hoecke et al. (2009) suggested that the toxicity was caused by an indirect effect of nutrient deficiency originating from adsorption of ammonium and/or phosphate to the NM surface. The authors conclude that the clustering of the NM aggregates around the algal cells, however, might have caused toxicity through local nutrient depletion.

Moreover, CNTs can adsorb both organic and inorganic compounds (Rao et al. 2007; Hoecke et al. 2009; Yang and Xing 2010), thereby potentially decreasing the total amount of nutrients in the algal culture medium and leading to toxicity by nutrient depletion (Tang et al. 2017). A summary of the toxicity pathways of NMs towards algae is presented in Figure 1.6.

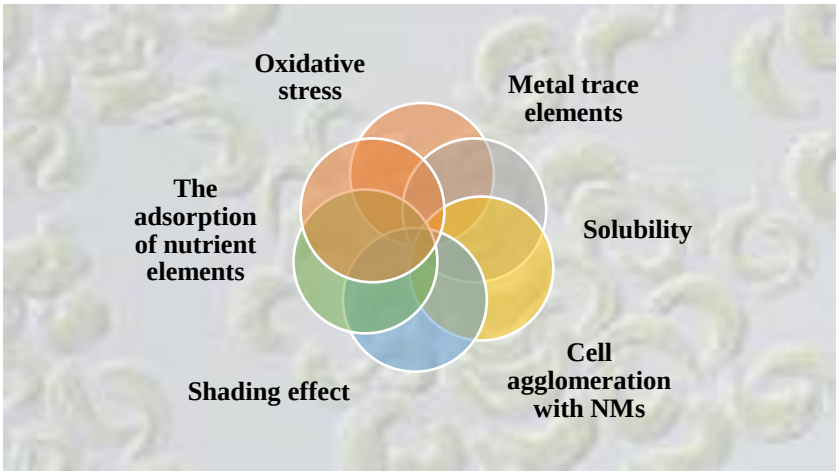


Figure 1.6: Scheme illustrating the several toxicity pathways of NMs towards algae.

1.9 The OECD 201 test guideline

The Organisation for Economic Cooperation and Development (OECD) has a role in providing advice regarding the safety of chemicals. The OECD produces a number of test guidelines, which are used in the safety testing of chemicals. One of these guidelines is the OECD Guideline 201, which is a 72-h assay that is used to measure the growth inhibition of microalgae and to assess the toxicity of chemicals to the aquatic environment.

This guideline is flexible in terms of species selection. It can involve using one of several species of microalgae or cyanobacteria, such as *Pseudokirchneriella subcapitata* (*Raphidocelis subcapitata*), *Desmodesmus subspicatus*, *Anabaena flos-aquae*, *Synechococcus leopoliensis* and *Navicula pelliculosa*. In contrast, in terms of growth medium, the OECD 201 guideline recommends the use of either alternative growth media – i.e. the OECD and the United States Environmental Protection Agency medium AAP. Under some circumstances, a modified medium can be used for testing metals and chelating agents or testing at different pH values. The test duration in this guideline is normally 72 h. However, test duration might be shorter or longer than the stated period, depending on specific considerations and relevance, and as long as assay conditions are met. Within a 72-h test period, the biomass in the control cultures must increase exponentially by a factor of at least 16. The mean coefficient of variation for the specific growth rates at each 24-h period must not exceed 35% in the control culture, and the value of the coefficient of variation for the average specific growth rate for the whole test period in replicate control cultures must not exceed 10% (for all species, with this recommended threshold value being 7% for *R. subcapitata*) (OECD, 2011).

1.10 Aims and objectives

The effects of NMs on the aquatic environment have been considered in a number of research articles (Klaine et al. 2008b; Selck et al. 2016; Zhu et al. 2019). However, there is still a distinct lack of knowledge about this subject, since only a few aquatic species and conditions have been tested to date (Fernandes et al. 2007; Leurquin et al. 2018; Palit, 2019). Moreover, there is still some to learn about conditions species, NMs dispersion methods, sonication period and the exposure time points. Therefore, it is important to expand the existing information on nanosafety by investigating the potential effects of these materials in the aquatic environment and on living organisms.

This research project had the overall aim of evaluating the ecotoxicology of Ag NMs and CNTs on the aquatic environment, given that these are some of the most widely used NMs to date, taking into consideration the method used to disperse the selected NMs. Toxicity to the microalga *R. subcapitata* was mainly assessed following the OECD 201 guideline. This aim was addressed via the objectives stated below:

- Investigation of the acute toxicity of Ag NMs and MWCNTs using standard methods to assess algal growth/production exposed to a different range of concentrations of these materials for 24, 48 and 72 h via evaluation of chlorophyll extraction (Chl a), optical density (OD), protein content and photosystem II activity (PSII).
- Assessment of differences between two standard methods of NM dispersion, bath and probe sonication. NM characterisation was carried out via DLS, TEM and visual examination. Different methods of sample preparation for TEM and EDX analysis were also tested.
- Assessment of the interaction and internalisation of NMs with *R. subcapitata* via examination by light microscopy (LM) and TEM.
- Finally, the activation of oxidative stress by used NMs was investigated via assessment of DCF assays and some of antioxidant defence systems in algae, including SOD, CAT activity and GSH level.

In the following chapters, the experimental work of this PhD project is presented. Acute toxicity of Ag NMs and MWCNTs is studied using different methods to assess algal growth exposed to a different range of concentrations of these NMs.

Initially, as outlined in Chapter 2, Ag NMs and MWCNTs were prepared in OECD 201 medium using two different methods; bath and probe sonication-induced dispersions were prepared and compared. DLS was used for Ag NM characterisation, as spherical NMs and TEM were used for both NMs using both sonication methods, as well as different methods of sample preparation: drop casting and plunge freezing of TEM samples. Then, EDX analyses were used. Moreover, visual examination was conducted for bath- and probe-sonicated MWCNTs.

Chapter 3 presents acute toxicity studies, in which *R. subcapitata* in OECD 201 medium was exposed to a different range of concentrations of these materials (both bath and probe sonicated) for 24, 48 and 72 h via evaluation of Chl a, OD, protein

content and PSII activity. Moreover, NM potential interference in the assays was investigated.

In Chapter 4, the interaction and internalisation of NMs with *R. subcapitata* was studied; this was carried out by exposing the algae to different concentrations of NMs (bath and probe sonicated) based on IC_{50} results (obtained from Chapter 3) for 72 h and examined by using LM and TEM.

Finally, in Chapter 5, *R. subcapitata* were used to assess the impact of Ag NMs and MWCNTs (both bath and probe sonicated) on the activation of oxidative stress by used NMs via assessment of DCF assay and some of the antioxidant defence systems found in algae, including SOD, CAT activity and GSH levels.

The rationale for this project is the testing and development of standard methods for the environmental safety assessment of nanotechnology. This project is an integral part of the work developed under the framework of the EU FP7 NanoReg project and currently ongoing OECD and ECHA developments. In this context, the results of this project will provide novel data, material and information, which will feed directly into the development of nanosafety regulatory approaches.

Chapter 2

NM characterisation

Chapter 2 **NM characterisation**

2.1 Introduction

The risk characterisation of NMs in the environment requires evaluation of both effects and exposure (Maynard et al. 2006; Crane and Handy 2007; USEPA 2007), and the possibility of exposure to NMs depends on their concentration in the environment, as well as their dispersibility and interaction with organisms. The prediction of the concentration of released NMs into the environment should be considered in relation to predicted releases of NMs, and this can be described by studying the fate and behaviour of NMs (Hassellöv et al. 2008). NMs have specific and unique properties which are different from conventional chemicals, however solely forecasting the concentration of NMs in the environment is not sufficient to determine the risk of these NMs in the ecosystem (Hassellöv et al. 2008).

The physicochemical properties of NMs are important for their behaviour and efficacy, but also for their effects on organisms, and thus their safety (Zhang et al. 2016). Therefore, when considering the safety of an NM, it is necessary to state not only its characteristics as a pristine NM but also how it may behave in relevant media, such as aqueous or solid matrices (Pleus 2012; Lin et al. 2014).

The most important physicochemical traits which can be used to assess NMs' safety, and which are thought to affect NMs' toxicity, are chemical composition, size, structure, shape, size distribution in aqueous media, surface area, solubility and agglomeration; these need to be considered when assessing toxicity (Murdock et al. 2008; López-Serrano et al. 2014). NM size directly affects the surface area and reactivity. Small NMs have a larger surface area compared to larger particles. This large surface area drives the particles to be more reactive, and to interact with other molecules in the medium (Midander et al. 2009). The different levels of toxicity from differently shaped NMs can also be explained by their surface area and reactivity, although these are not the sole causal elements. Surface coating and the tendency to agglomerate also affect the toxicity of NMs by changing their surface reactivity. Agglomeration commonly affects the surface of NMs, and leads to lower interactions with surrounding molecules (Li and Lenhart 2012).

Characterisation of NMs can provide information on the evolution of NMs in the test medium, and also on the relationship between NM properties and observed effects in *R. subcapitata*. Moreover, the relationship between NM characteristics and their toxic

effects is still unclear, and for this reason it is advisable to measure a range of potentially significant aspects, such as size, shape, and surface charge amongst others (Jiang et al. 2009).

2.1.1 *Interaction between NMs and biological environments*

Since the use of NMs is increasing in various types of end products and applications, the toxicological effects of NMs can potentially be compared (and extrapolations made) based on their characteristics, which again relate to their fate and behaviour in the environment (Hassellöv et al. 2008). Moreover, the introduction of NMs into natural environments leads to interactions with substances or molecules of biological origin, leading to agglomeration, coagulation and nonspecific absorption, as a result of intermolecular interactions happening at the interfaces of NMs with biomolecules and other biological substances (Nel et al. 2009).

As described previously, key NM properties include chemical composition, shape, surface geometry and crystallinity, porosity, heterogeneity and hydrolytic stability, and features such as surface charge, dissolution, hydration, size distribution, dispersion stability, agglomeration and aggregation. Some of the properties are controlled by ionic strength, pH, temperature and the presence of biological or organic macromolecules (Oberdörster et al. 2005; French et al. 2009; Nel et al. 2009; Hull and Bowman 2018).

Consequently, in order to assess NM fate and behaviour, an assessment must be made of the NMs of interest in the relevant test medium, to evaluate dissolution, suspension, agglomeration, etc. (Hull and Bowman 2018). In this chapter, the characterisation and behaviour of NMs in the test conditions are investigated.

2.1.2 *Dispersion*

The way NMs are distributed in aqueous medium depends on the ionic strength of the solution and its pH, as well as primary size and surface chemistry of the particles (Jiang et al. 2009). In biological media other elements have to be taken into account; typically, NMs used for cellular delivery are coated with a functional polymer and delivered in a liquid containing biomacromolecules and simple amino acids, vitamins and serum proteins, while also being protected by ionic salts, all of which may combine to influence the hydrodynamic behaviour and biointeractivity of the NMs (Lynch and Dawson 2008; Nel et al. 2009). The size, including the degree of

agglomeration, is sometimes hard to assess even after ultrasonication has been carried out to break up agglomerates (Hondow et al. 2015).

CNTs can be dispersed in water when coated with adsorbed surfactants, preferably with those that have relatively high HLB (Hydrophyle–Lyphophyle Balance) (Vaisman et al. 2006b). This non-covalent method is straightforward and classically employed to disperse both organic and inorganic particles in aqueous solutions (Vaisman et al. 2006b). The nature of the surfactant, its concentration, and the type of interaction are known to play a crucial role in the phase behaviour of classical colloids (Rosen 2004) as well as CNTs (Cui et al. 2003; Shvartzman-Cohen et al. 2004; Yurekli et al. 2004; Hertel et al. 2005). Knowing the surface charge of CNTs in different media is essential for understanding the interaction mechanism (e.g. adsorption) with ionic surfactants, and to predict the colloidal stability of CNT suspensions. While zeta potential analysis of MWCNT has shown the tubes to be negatively charged (-40 mV) in distilled water with the addition of sodium dodecyl sulphate (SDS) in a study by Jiang et al. (2003), other researchers have demonstrated insufficient "debundling" (separation from bundles) power of the anionic surfactant SDS due to charger repulsion (Vaisman et al. 2006a), whereas others reported a uniform colloidal dispersion of SDS-coated CNTs (Rosen 2004).

The effect of a head-group charge was investigated for various CNT-based systems (Chatterjee et al. 2005; Vaisman et al. 2006a), revealing no clear conclusion on the superiority of either cationic or anionic surfactants in dispersing the tubes. It seems that the adsorption mechanism to the CNTs of ionic surfactants, which is promoted by electrostatic interactions with a CNT surface, is heavily controlled by the purification process and wall-functionalisation of the tube, which in turn determine its surface charge (Vaisman et al. 2006b). CNTs were ultrasonicated for 5 min and various dispersants were added to improve the dispersion of nanotubes within aqueous solutions such as DTAB, SDS, Pluronic L-35, L-44, and P-65 (Vaisman et al. 2006a).

The level of agglomeration and changes in the surface properties of NMs in suspension are influenced by the method of dispersion (Pradhan et al. 2016). Based on particle characteristics and selected sonication type (bath, probe, stirring) and parameters (e.g. time, temperature, sonication amplitude), suspended NMs will to a certain extent transform, dissolve, agglomerate and interact with the surrounding medium components (Cohen et al. 2013).

Knowing what happens in the transformation of NMs because of sonication is a useful factor concerning NM dispersions. Cohen et al. (2015) and Hartmann et al. (2015) indicate that an understanding of how sonication affects particle characteristics (e.g. size, zeta potential) is hence essential, as it potentially has a large influence on the toxic response of the NMs (Midander et al. 2009; Nel et al. 2015).

2.1.3 *Size and shape of NMs*

Size and shape are important parameters for the selection of NMs (Zhang et al. 2017a), and affect their use and performance in different applications (Culver et al. 2016; Rampersaud et al. 2016). It is conventional to consider any particulate materials between 1 and 100 nm as NMs (European Commission. 2011b). As such, size is the most important parameter when defining NMs (Powers et al. 2007). It is also accepted, and indeed has been verified by a series of experiments, that smaller-sized particulate matter, certainly below 100 nm, tends to lead to higher toxicity (Kloepfer et al. 2005; Morones et al. 2005; Pal et al. 2007; Choi et al. 2008). Furthermore, Pal et al. (2007) found that Ag NMs undergo shape-dependent interaction with the gram-negative bacteria *E. coli*. While various NMs do not have a regular shape, NM size is typically expressed in terms of the equivalent spherical diameter (Powers et al. 2007).

Identifying NM size is a crucial factor in NM characterisation. NM size can drive how and where the NM interacts with the organism or cell (Borm et al. 2006), the ability of the organism or cell to clear foreign NMs' (Renwick et al. 2001) fate, behaviour, translocation or accumulation of NMs, and these can also affect the surface reactivity and solubility of NMs (Powers et al. 2007). The uptake of NMs by cells is influenced by the size of NMs, and due to agglomeration of NMs, size of cluster will be larger than the original NMs, and this may make interactions of NMs with living material, or uptake, more complex (Benne et al. 2016; Silva et al. 2016). Moreover, Powers et al. (2007) indicate that size should reasonably be the first parameter to consider when characterising NMs for toxicity studies.

2.1.4 *Zeta potential*

The surface charge of NMs in suspension can be assessed by determining the zeta potential. If the surface of NMs is charged this can lead to attraction of a thin layer of ions of opposite charge. These then travel with the NM when diffusing through the suspension. The zeta potential of the NMs is the electric potential at the boundary of

the two layers mentioned, and its values are normally in the range between -100 mV and +100 mV. The value of the zeta potential can be used to predict colloidal stability (NanoComposix 2012).

NMs with zeta potential values greater than +25 mV or less than -25 mV typically have high degrees of stability (NanoComposix 2012). Dispersions with a low absolute zeta potential will eventually likely aggregate due to Van Der Waals interparticle attractions. Determination of zeta potential is important to understand the state of the surface of the NMs and to be able to predict the long-term stability of the NM suspension.

Knowledge of the surface charge is necessary so that the biological response can be better understood (Fröhlich 2012). A study was conducted by El Badawy et al. (2011) on the surface-charge-dependent toxicity of 4 types of Ag NMs (uncoated H_2-Ag NMs, citrate-coated Ag NMs (Citrate-Ag NMs), polyvinylpyrrolidone-coated Ag NMs (PVP-Ag NMs), and branched polyethyleneimine coated Ag NMs (BPEI-Ag NMs)) to *Bacillus* species. The results showed that positive surface charge often led to increased toxicity. In other words, the more negative Citrate-Ag NMs were the least toxic, whereas the positively charged BPEI-Ag NMs were the most toxic NMs. Although knowledge of pristine NMs' properties and understanding of fate and behaviour in relevant media are essential to understand and predict toxicity, it is also important to realise that different cell types will potentially react differently when in contact with NMs in their exposure medium (Albanese and Chan 2011).

2.1.5 *Characterisation techniques*

There are different methods to characterise NMs, and these are selected based on the objectives of the study and the availability of techniques. They include: dynamic light scattering (DLS); X-ray diffractometry (XRD); X-ray photoelectron spectroscopy (XPS); Fourier transform infrared spectroscopy (FTIR); scanning electron microscopy (SEM); atomic force microscopy (AFM); transmission electron microscopy (TEM), and others (Handy et al. 2008b; Hassellöv et al. 2008; Sapsford et al. 2011; López-Serrano et al. 2014; Gurunathan et al. 2015b; Zhang et al. 2016). In this study, characterisation of Ag NMs and MWCNTs was necessary for the evaluation of their fate in experimental media and understanding of exposure conditions that may lead to toxic effects. Each analytical technique has a variety of principles and applications,

and the approach adopted in this research study for the characterisation of Ag NMs and MWCNTs is detailed below.

2.1.5.1 Dynamic light scattering (DLS)

DLS is a method used to identify particle size in regards to hydrodynamic diameter. It is important to note the difference between hydrodynamic diameter and absolute particle diameter; hydrodynamic diameter is the absolute diameter of the particle plus the layer of electrostatically bound ions that move with the particle in an aqueous medium (Figure 2.1). The size obtained from DLS is usually larger than that obtained by TEM, and this might be influenced by Brownian motion (Malvern Instruments Ltd 2013; Zhang et al. 2016). DLS is a non-destructive method, from which an average diameter of NMs dispersed in liquids can be obtained. The advantage of this approach is that a large quantity of materials can be tested at the same time, although there are some sample-specific limitations (Lange 1995; Kou et al. 2012).

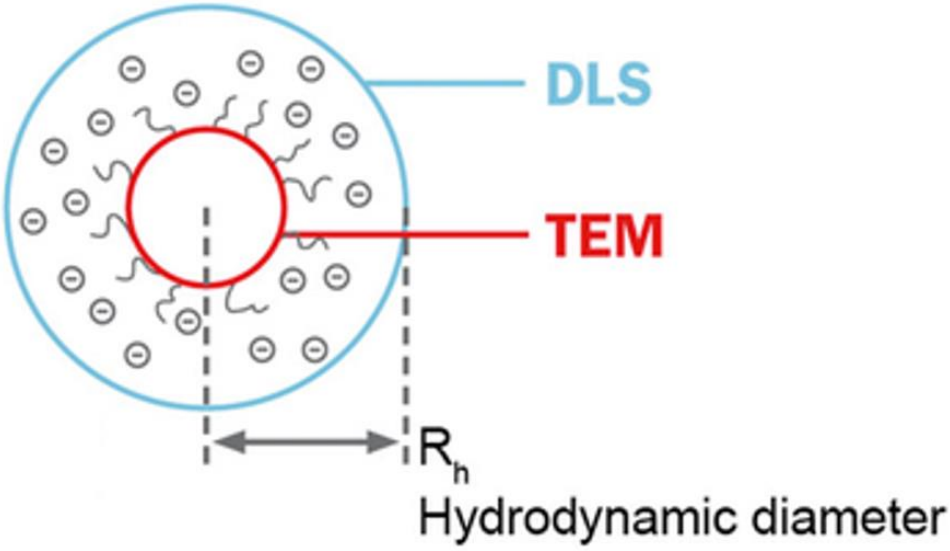


Figure 2.1: The difference between hydrodynamic diameter and absolute particle diameter. DLS is referred to the dynamic light scattering and TEM is referred to the transmission electron microscopy, adapted from (Fritsch, 2018).

DLS is the most common technique for characterising NMs (Jans et al. 2009; Zanetti-Ramos et al. 2009; Khlebtsov and Khlebtsov 2011). The DLS technique relies on Rayleigh scattering from the suspended NMs that undergo Brownian motion (Fissan et al. 2014). Furthermore, light interacts with particles in the DLS method (Tomaszewska et al. 2013). Following this, scattered light intensity is analysed and determines the hydrodynamic particle size (Berne and Pecora 2000; Koppel 2003; Dieckmann et al. 2009). This method can be used to determine the size distribution of

particles ranging between 1 nm and less than 1 µm in suspension (Patri et al. 2006; Sapsford et al. 2011; Lin et al. 2014).

During DLS analysis, laser illumination of the particles takes place and fluctuations in light intensity by light scattering caused by the particles are recorded. Smaller particles move faster, therefore the speckle pattern caused by the laser hitting the particles will fluctuate more rapidly for small particles and correlation (similarity of intensity signals) over time will decrease faster than for larger particles. By using the correlation function, a size distribution can be generated by the Zetasizer (Malvern Instruments Ltd 2013). The Zetasizer software generates the Zeta average (Zavg), which is a mean value of particle size based on the intensity of the measured sample (Deng et al. 2013). It is also important, if possible, to characterise particle size during the test period, as agglomeration can occur over time (Hoecke et al. 2008).

Hydrodynamic diameter (measured by DLS) is sensitive to ionic strength and other medium-specific factors (Jiang et al. 2009). One of the studies which evaluated Ag NMs particle size using DLS, hydrodynamic diameter has been found to be larger than the pristine particle size of 15 nm described by Klein et al. (2011). Hydrodynamic diameters of 12–140 nm have been reported (Kermanizadeh et al. 2013; Losasso et al. 2014; Mallevre et al. 2014; Donnellan et al. 2016), which may vary due to medium composition or dispersion protocol.

2.1.5.2 Transmission electron microscopy (TEM)

To characterise NMs, TEM is the most commonly used method to obtain quantitative measurements of particle and/or grain size, size distribution, and morphology (Williams et al. 1998; Joshi et al. 2008; Lin et al. 2014). There are two advantages of TEM compared to SEM: TEM offers better spatial resolution and the capability for additional analytical measurements (Williams et al. 1998; Hall et al. 2007; Lin et al. 2014). In contrast, there are also disadvantages, which are the necessary high vacuum and a thin sample section (Hall et al. 2007; Joshi et al. 2008; Lin et al. 2014), and an important aspect is that sample preparation is very time consuming (Zhang et al. 2016).

2.1.5.3 EDX

EDX is short for Energy-dispersive X-ray spectroscopy, used as an additional analytical tool in some electron microscopes (Rasmussen et al. 2014). EDX can detect elements with an atomic number larger than 5 (e.g. carbon and heavier elements), and

retrieves information on spatial (two-dimensional) atom distribution well. Even so, the suitability for NMs of complex composition, in complex matrices, and for large agglomerates is not assured (Rasmussen et al. 2018).

2.1.6 *NM properties*

Silver NMs (NM300k) and multi-wall carbon nanotubes (NM400) were obtained from the Joint Research Centre of the European Commission (JRC). The JRC Nanomaterials Repository was established for research purposes in support of the OECD.

2.1.6.1 Ag NMs (NM300k)

Ag NMs is an aqueous dispersion of Ag NM with stabilising agents, consisting of 4% w/w of each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan mono-laurate (Tween 20) (Klein et al. 2011). Particle size distribution is narrow (derived from scanning electron microscopy, transmission electron microscopy and an analysis of NM tracking), with the majority of particles having a diameter of around 15 nm, and a much smaller proportion around 5 nm (Klein et al. 2011). The vials contain an argon atmosphere to improve sample stability.

A number of studies have characterised Ag NMs properties in some test media. Völker et al. (2013) found that in OECD M4 medium (*Daphnia sp*.), Ag NMs showed a DLS-derived particle size of 57.6 ± 1.2 nm and surface charge of -17 ± 0.57 mV. Kermanizadeh et al. (2013) found that in cell culture medium, Ag NMs showed 3 DLS-derived particle size classes of 12, 28, and 114 nm in Milli-Q water and C3A medium after 24 h. Wang et al. (2012) found that in unspecified media, Ag NMs exhibited a DLS-derived zeta potential of -10 to -14 mV and a wide particle size distribution (100–200 nm). Sorensen and Baun (2014) found that Ag NMs had a DLS-derived size of 106 (±53) nm and zeta potential of -21 mV at 0 h in ISO algae medium. A summary of literature reviewed regarding the physicochemical properties of Ag NMs Ag NMs is presented in Table 2.1.

Table 2.1: Summary of the literature reviewed regarding physicochemical properties of Ag NMs

No.	NMs	Medium	Diameter (nm)	Zeta potential (mV)	Additional information	Reference
1	Ag NMs	Milli-Q water	<20	ND	Surface capping by polyoxyethylene glycerol trioleate and Tween 20. This report as standard and reference for Ag NMs.	(Klein et al. 2011)
2	Ag NMs	Test medium (zebra fish)	95–115 200–210	-10.3 -13.9	The particle size of the Ag NMs in the test medium had a wide distribution.	(Wang et al. 2012)
3	Ag NMs	Human hepatoblastoma C3A	12, 28, 114	ND	Mainly euhedral NM; minor fractions had either elongated or sub-spherical morphology.	(Kermanizadeh et al. 2013)
4	Ag NMs	OECD M4 medium	57.6 ± 1.20	-17.0 ± 0.57	The aggregation state of the particles did not allow for definite conclusion on the NMs' toxicity.	(Völker et al. 2013)
5	Ag NMs	Mueller Hinton broth (MHB)	5–19	ND	Most particles were spherical with sporadic presence of regular polygonal NMs. No differences in Ag NM size or shape after incubation in the culture medium MHB with no aggregates. The contaminating elements were negligible.	(Losasso et al. 2014)
6	Ag NMs	ISO algae medium	106 ± 53	-21 ± 4.3	The morphology of Ag NMs was close to spherical. Surface capping by polyoxyethylene glycerol trioleate and Tween 20.	(Sorensen and Baun 2014)
7	Ag NMs	Middlebrook 7H9	53.34 ± 3.1	-8.77 ± 2.9	Agglomeration occurred in the growth medium. Limited agglomeration of Ag NMs was observed by TEM.	(Donnellan et al. 2016)
9	Ag NMs	Milli-Q water	33.8–71.7 12.5 ± 4.1 by TEM	-7.32	DLS measurements indicated a larger mean particle size compared to TEM images. Agglomerates were present and varying in size.	(Kleiven et al. 2018)

2.1.6.2 MWCNTs (NM400)

NM400 is a multi-walled carbon nanotube (MWCNT) shown as a black powder in amber-coloured vials containing 250 mg or less under argon atmosphere (Rasmussen et al. 2014). The diameter is derived from small-angle X-ray scattering (SAXS), and transmission electron microscopy (TEM), with the majority of nanotubes having a diameter of around 30 nm, and a length of around 5 µm (Rasmussen et al. 2014). In addition, NM400 shows a great variation in the content and type of impurities.

Previous studies have provided information on the characteristics of NM400 in different test media. Kermanizadeh et al. (2012) have characterised NM400 properties and found that in Milli-Q water, NM400 revealed misshapen and tangled MWCNTs (10–20 walls) and that the diameter was in the range of 5–30 nm, with a length in the range of 0.7–3 µm. Some CNTs has one cap and some cases has several caps. Moreover, Fe and Co catalysts (6–9 nm, average 7.5 nm) were found inside the NM400 tubes. Hougaard et al. (2013) found that NM400 had a length of 295 ± 234 nm (geodesic) and a diameter of 10 ± 3 nm in distilled water. Moreover, NM400 was found highly bent by MWCNTs with some chemical compositions, such as Al, Fe and Co. Sauer et al. (2015) used TEM to characterise NM400 suspended in BSA-containing medium or Curosurf phospholipids. The average diameter was 30 and 416 nm for BSA and Curosurf, respectively, and the length was 1.5 µm (Sauer et al. 2015). A summary of the literature reviewed regarding the physicochemical properties of MWCNTs (NM400) is presented in Table 2.2.

Table 2.2: Summary of the literature reviewed regarding the physicochemical properties of MWCNTs (NM400)

No.	NMs	Medium	Diameter (nm)	Length (µm)	Additional information	Reference
1	NM400	Human hepatoblastoma C3A	30	5	Short entangled.	(Kermanizadeh et al. 2013)
2	NM400	Fetal bovine serum (FBS)	5–35	0.7–3	Impurities: Al, Fe.	(Vietti et al. 2013)
3	NM400	Water Saline (Mice) RPMI (Endothelial Cells)	198 ± 85 124 ± 50 143 ± 59	ND	Appeared to consist of entangled, irregular, and bent nanotubes.	(Cao et al. 2014)
4	NM400	Milli-Q water	15	<1.5	Contained a fraction of µm-sized particles (corundum crystals).	(Rasmussen et al. 2014)
5	NM400	Sterile Baxter water (THP-1 cell line)	11 ± 3	0.846 ± 0.446	Elemental impurities detected: >0.01%, Al, Fe, Na, S. Bundles and individual rope-like structures.	(Öner et al. 2016)
6	NM400	EMEM (fish lever)	515.1 ± 18.	0.639–4.917	Lake of viability between measurements at 0 and 24 h indicate the stability of the suspension.	(Bermejo-Nogales et al. 2017)
7	NM400	Milli-Q water (algae)	11 ± 3	0.84 ± 0.4	Unstable dispersions.	(Farkas and Booth 2017)
9	NM400	DMEM/F12 (human bronchial epithelial cell lines)	~0.4–3.5	ND	DLS is not an ideal method to determine hydrodynamic diameter for CNTs, however DLS data can be used as representative of aggregation state over time.	(Ghosh et al. 2018)
10	NM400	Distilled water	10 ± 3	295 ± 234	Highly bent MWCNTs, with impurities (Al, Fe, Co, and Na).	(Hougaard et al. 2013)

2.1.7 *Aim*

The main aim of this chapter is to determine the physicochemical properties and behaviour of Ag NMs (NM300k) and MWCNTs (NM400) in the test medium (OECD 201), comparing two dispersion methods, bath and probe sonication. The results of this work will provide information necessary for the interpretation of the safety and hazard data. Three different techniques were used in this study: dynamic light scattering (DLS), transmission electron microscopy (TEM) and visual examination (Figure 2.2).

2.1.7.1 Aim of DLS study

This study was conducted to examine the level of agglomeration of Ag NMs (as a spherical NM) in OECD 201 medium over the time period of 0, 24, 48 and 72 h. Since the NMs in this study were dispersed using two different sonication methods, the experiment was conducted to compare the behaviour of Ag NMs in the test medium using bath sonication, as well as probe sonication, in order to establish the influence of the sonication on the behaviour of Ag NMs in the test medium. The null hypotheses were that the level of agglomeration of Ag NMs would not be influenced by the sonication method, bath or probe, nor by the time period.

2.1.7.2 Aim of TEM study

The aim of the TEM study was to visualise Ag NMs and MWCNTs, dispersed using two different types of sonication method (bath and probe), to examine the level of agglomeration in the test medium (OECD 201), and compare between the two sonication methods as well as the approach taken to prepare the samples for TEM analysis.

The null hypotheses were that the level of agglomeration of Ag NMs and MWCNTs would not be influenced by the sonication method, and that the procedure used to prepare the sample for TEM analysis (drop cast or plunge freeze) does not affect the results.

2.1.7.3 Aim of visual examination study

The aim of this study was to assess MWCNT stability, as an aqueous suspension, in Milli-Q water and OECD 201 medium, based on visual examination. The null

hypothesis was that the level of settling MWCNTs would not be influenced by bath sonication nor by probe sonication in the test medium.

2.1.7.4 Aim of stability study

The objective of this study was to understand what is likely to influence of sonication process on behaviour of MWCNTs in Milli-Q water and OECD medium. The null hypothesis was that the stability of MWCNTs in medium would not be influenced by bath sonication or by probe sonication.

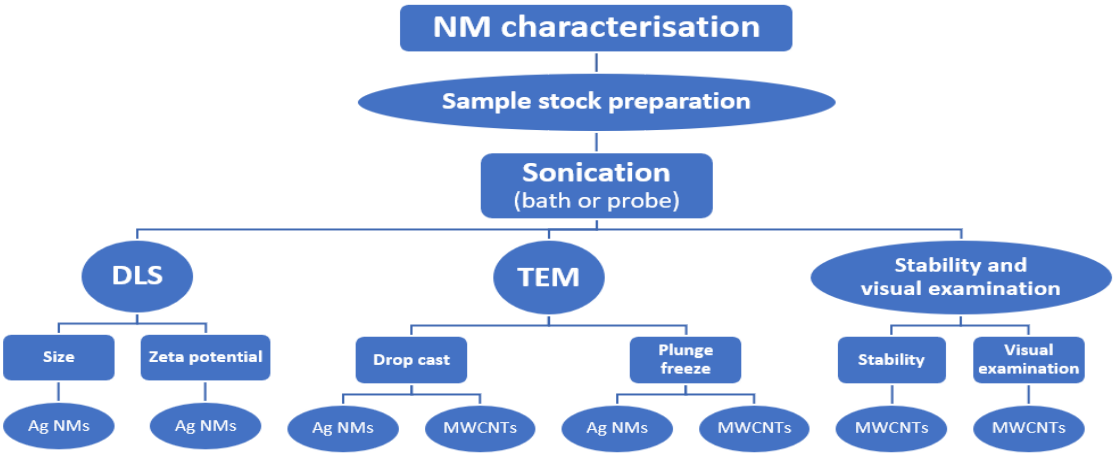


Figure 2.2: Scheme of the different methods used for characterisation of NMs in this research study.

2.2 Methodology

As part of the physicochemical characterisation, the sample preparations were done using bath sonicator (Pulsatron 325, Kerry) (2 × 8 min) with ~10 seconds manual shaking in between, following the protocol developed for ENPRA (Jacobsen et al. 2010) or by probe sonication (14.11 min) based on the standard operating procedure for NanoReg (Booth and Jensen 2015).

2.2.1 *Preparation of glassware*

All glass test containers were acid washed prior to use by soaking them in a solution of 50% nitric acid (HNO_3) for a minimum of 15 min. Thereafter, the material was rinsed thoroughly 3–4 times with Milli-Q water, then the flasks were allowed to dry completely. All the work was performed in the fume hood following the required safety precautions.

2.2.2 *OECD 201 medium*

OECD 201 growth medium was used in all experiments. The OECD medium was made up following the procedure described in OECD Guideline 201 (OECD, 2011). Milli-Q water was used to prepare OECD 201 medium. In the preparation of this medium, four stock solutions were initially prepared (Table 2.3). A volume of 10 mL of macronutrients, 1 mL of iron, 1 mL of trace elements and 1 mL of bicarbonate stock solution were added into Milli-Q water to prepare 1 litre of OECD 201 medium.

Table 2.3: Composition of the growth nutrients in OECD 201 medium (OECD 2011)

Stock	Nutrient	Concentration in the stock solution
Stock solution 1: macronutrients	NH_4Cl	1.5 g/L
	$MgCl_2.6H_2O$	1.2 g/L
	$CaCl_2.2H_2O$	1.8 g/L
	$MgSO_4.7H_2O$	1.5 g/L
	KH_2PO_4	0.16 g/L
Stock solution 2: iron	$FeCl_3.6H_2O$	64 mg/L
	$Na_2EDTA.2H_2O$	100 mg/L
Stock solution 3: trace elements	H_3BO_3	185 mg/L
	$MnCl_2.4H_2O$	415 mg/L
	$ZnCl_2$	3 mg/L
	$CoCl_2.6H_2O$	1.5 mg/L
	$CuCl_2.2H_2O$	0.01 mg/L
	$Na_2MoO_4.2H_2O$	7 mg/L
Stock solution 4: bicarbonate	$NaHCO_3$	50 g/L
	$Na_2SiO_3 \cdot 9H_2O$	

2.2.3 *Nanomaterials*

The NMs used in this study (Ag NMs and MWCNTs) were obtained from the European Commission Joint Research Centre (JRC). Stock suspensions of 100 mg/L were prepared in 20 mL glass vials. Suspensions were prepared fresh daily prior to each experiment.

2.2.4 *NM stock suspension preparation*

This section describes the preparation of the NM suspensions. Exposures were prepared with control (OECD medium only without algae cells) and 5 concentrations of NMs, with 3 replicates (in an approximate volume of 53 mL each).

For the preparation of Ag NMs, 49.2 mg of Ag NMs dispersion was suspended in 50 mL OECD 201 medium to create the stock suspension B (100 mg/L). Then the

suspension was sonicated at 38 kHz ± 10% in a bath sonicator (Pulsatron 325, Kerry) twice for 8 min each time, with ~10 seconds of manual shaking in between. Probe sonication took place for 14.11 min (Jacobsen et al. 2010). A volume of 5 mL from stock suspension B was diluted with 50 mL OECD medium 201 in order to prepare stock suspension C (10 mg/L). A volume of 5 mL from stock suspension C was diluted with 50 mL OECD 201 medium in order to prepare stock suspension D (1 mg/L). The concentrations used were 20, 50, 80, 150, 200 and 300 µg/L for Ag NMs.

The MWCNT suspension was prepared by dispersing 10 mg of MWCNTs in 100 mL OECD 201 medium by either bath or probe sonication, following the same procedure as for silver NMs, in order to prepare the stock suspension. This was then diluted in the appropriate medium concentration in order to prepare the required exposure concentrations without algal cells. The concentrations required were 1, 5, 25, 45, 70 and 100 mg/L.

2.2.5 *Sonication*

A stock suspension of NMs (100 mg/L) was prepared by suspending the NMs in Milli-Q water in 20 ml glass vials. The suspensions were sonicated by bath sonication for 16 min (twice for 8 min each) based on a protocol developed for ENPRA (Jacobsen et al. 2010) or by probe sonication according to the NanoReg protocol (Booth and Jensen 2015). The delivered acoustic power is calculated according to the equation:

$$Pac\ (Watt) = \frac{\Delta T}{\Delta t}\ MCp \qquad (1)$$

where: $\frac{\Delta T}{\Delta t}$ is the slope of the regression curve, *T* is the temperature (K), *t* is the time (seconds), *Cp* is the specific heat of the liquid (4.18 J/g*K for water), and *M* is the mass of liquid (g).

Based on the above equation, NM suspensions must be sonicated for 14.11 min to deliver total energy of 7,056 ± 103 J. The sonication time was determined experimentally following an internal NanoReg standard operating procedure for probe sonicator calibration of delivered acoustic power and de-agglomeration efficiency for ecotoxicological testing (Booth and Jensen 2015). The main goal of this calibration was to provide sufficient energy to de-agglomerate NM suspensions for toxicological testing and to obtain a uniform quality of NM suspension before using it in

toxicological studies. Following sonication, NM suspensions were diluted in OECD 201 medium to the required concentrations.

2.2.6 *DLS*

DLS (Zetasizer Nano Series, Malvern Instruments, Malvern, UK) was used to monitor Ag NM hydrodynamic diameter and zeta potentials in an algal medium (OECD 201 medium). The samples were analysed in a Folded Capillary Cell (DTS1070, Malvern Instruments). The measurements of the Ag NMs were performed at 23 °C, following the manufacturer's protocols (Malvern Instruments Ltd 2013). Ag NMs suspensions were freshly prepared from stock suspensions (bath or probe sonicated), then subsequently diluted in OECD 201 medium.

During the experiment, while the samples were incubated in the orbital shaker under the same conditions as used in the acute toxicity test (225 RPM, with continuous fluorescent light ~120 µmol/m^2.s, at 23° C ± 2° C), MWCNTs were not analysed by DLS, as only spherical particles are suitable for DLS measurements (Malvern Instruments Ltd 2013).

2.2.6.1 Hydrodynamic size measurement

The particle size of Ag NMs was determined using a particle size analyser (Malvern Instruments, Malvern, UK). The particle size was obtained by measuring the time-dependent fluctuation of the scattering of laser light by the NM undergoing Brownian motion. Approximately 1.5 mL of concentrations of 20, 50, 80, 150, 200, 250 and 300 µg/L Ag of Ag NMs, as well as the blank control (just OECD 201 medium), were injected into a plastic measuring cuvette (DTS1070, Malvern Instruments) and determined in triplicate at t=0, 24, 48 and 72 h.

2.2.6.2 Zeta potential measurement

The zeta potential of Ag NMs was determined by means of the zeta potential analyser (Malvern Instruments, Malvern, UK). The measurement of zeta potential was based on the direction and velocity of particles under the influence of a known electric field. Ag NMs, as well as blank control (just OECD 201 medium), was injected into a plastic measuring cuvette (DTS1070, Malvern Instruments) and determined in triplicate at t=0, 24, 48 and 72 h.

2.2.7 *TEM*

The morphology of the studied NMs was determined by TEM, for which the suspension, containing NMs (Ag NMs or MWCNTs), was suspended in algal medium (OECD 201) at 10 mg/L. Two different techniques – drop cast and plunge freezing – were used in this study to prepare the TEM samples. All TEM work was done at the School of Chemical and Process Engineering, at University of Leeds, UK.

2.2.7.1 Drop casting of TEM samples

After preparing the appropriate NM concentrations (10 mg/L Ag NMs and 10 mg/L MWCNTs), a 3.5 µL droplet of the suspension was placed on a copper TEM grid coated with a carbon support film (Agar Scientific Ltd. UK). The drop of the suspension was allowed to evaporate at room temperature (in an agar plate to avoid contamination) overnight before analysis (Kalman et al. 2015). NMs were assessed on an FEI Tecnai F20 FEG-TEM operated at 200 kV fitted with a Gatan Orius Charge-Coupled Device (SC600A CCD) camera.

2.2.7.2 Plunge freezing of TEM samples

After preparing the appropriate concentrations of NMs (10 mg/L Ag NMs or MWCNTs), a 3.5 µL droplet of the suspensions was placed on a copper TEM grid coated with a carbon support film (Agar Scientific Ltd. UK). Then, the sample was immediately plunge frozen in liquid ethane (C_2H_6). Samples were then stored in liquid nitrogen (LN_2). The samples were examined by TEM and were subsequently transferred cold into a Gatan 626 cryo-transfer holder, in which they were cooled by liquid nitrogen (White et al. 1998; Hondow et al. 2012).

2.2.7.3 EDX analysis

TEM EDX analysis was used to confirm the identity of Ag NMs, directly, or MWCNTs through the presence of trace metal contaminants such as Fe on the MWCNTs' walls. The EDX was performed using an Oxford Instruments 80 mm^2 Silicon Drift Detector (SDD) fitted to the TEM and running INCA software (University of Leeds, UK).

2.2.8 *Visual examination of MWCNTs*

This study was carried out to determine the stability of MWCNTs in MilliQ-water and OECD 201 medium. The MWCNTs (100 mg/L) were bath sonicated (for 2 × 8 min)

or probe sonicated (for 14.11 min) in MilliQ-water or OECD 201 medium. A volume of 20 mL of this suspension was immediately transferred into glass vials (Figure 2.25 and 2.26) for visual examination of the stability of MWCNTs (NM400) in MilliQ-water, as well as in OECD 201 medium, at 0, 0.25, 1 and 24 h.

2.2.9 *Stability of MWCNTs in aquatic environments*

This study was carried out to determine the stability of MWCNTs in aquatic environments, specifically MilliQ-water and OECD 201 medium. The MWCNTs (100 mg/L) were bath sonicated (for 2 × 8 min) or probe sonicated (for 14.11 min) in Milli-Q water or in OECD 201 medium. A volume of 2.7 mL of this suspension was immediately transferred into a cuvette (1 cm path length) and absorbance measurements performed at 660 nm (Zhang et al. 2014) at intervals of 5 min for 120 min, then there was one additional reading at 24 h using the spectrophotometer (Jenway model 6715). The objective of this study was to improve the understanding of the factors that may influence the fate and behaviour of MWCNTs in a diverse range of real environmental media.

2.2.10 *Statistical analyses*

DLS data were analysed in Minitab 18 statistical software. Data were found to be normally distributed, and so parametric statistics were used. Significant differences were determined using one-way or two-way ANOVA with post hoc multiple comparisons (Tukey) at the level of (p<0.05) (to identify significant differences between 2 groups). For the sake of clarity and tidiness, the significant differences signs on the error bars have not been included in some of the graphs.

2.3 Results

2.3.1 *DLS*

2.3.1.1 Hydrodynamic diameter

The hydrodynamic diameter was measured immediately after sample preparation, as well at 24, 48 and 72 h. The summary of the hydrodynamic diameter results obtained for the Ag NMs using both sonication methods (bath and probe) by DLS is shown in Figure 2.3.

At 0 h, the results showed that the mean hydrodynamic diameters across all exposure concentrations for both types of sonication, bath and probe, were around 100 nm and

≤95 nm, respectively (Figure 2.3 A). In the control sample (OECD 201 medium only) there was no sign of a hydrodynamic diameter, as no particles were present in the samples analysed. For the Ag NMs, at 0 h, the average hydrodynamic diameter (Z-average) for all tested concentrations was in the ranges of 92.4–109.96 nm and 78.06–95.13 nm, for bath and probe sonication, respectively. Moreover, there were no significant differences between the measurements for each type of sonication (ANOVA, Tukey's post hoc test). Indeed, in terms of sonication type, there was no significant difference between bath and probe sonication for all concentrations of Ag NMs at 0 h (Figure 2.3 A).

After first measurements at 0 h, the samples were incubated the orbital shaker for 72 h, under the same conditions as the toxicity experiment, where the Ag NMs particle hydrodynamic diameters for all tested concentrations were found to be between 119.00 and 173.43 nm, and between 95.4 and 154.9 nm for bath and probe sonication, respectively. Incubation for 24 h caused a slight increase in the hydrodynamic diameter in both types of sonication (bath and probe) compared to the hydrodynamic diameter of the Ag NMs at 0 h. However, when comparing all the tested concentrations of Ag NMs using the same sonication process (bath or probe), no significant differences were detected between the two sonication methods across the tested concentrations. Moreover, in terms of sonication type, while the hydrodynamic diameter of bath sonicated Ag NMs was higher than in case of the probe sonicated Ag NMs at 24 h, however, these values are not significantly different (Figure 2.3 B). At 48 h, the hydrodynamic diameter measurement for Ag NMs in the OECD 201 medium is displayed in Figure 2.3 C, which shows the hydrodynamic diameters to be between 143.66 and 180.7 nm, and between 122.06 and 179.63 nm for all tested concentrations for bath and probe sonication, respectively. Incubation for 48 h caused a slight increase in the hydrodynamic diameter in both types of sonication (bath and probe) compared to the hydrodynamic diameter of Ag NMs at 0 and 24 h. Nevertheless, when comparing all tested concentrations of Ag NMs using the bath-sonication process, there was no significant difference between the tested concentrations. However, the probe-sonication method significantly decreased the hydrodynamic diameter of 80 µg Ag/L of Ag NMs compared to 20, 50, 80 and 300 µg Ag/L. Furthermore, there was no significant difference found between the sonication types (bath and probe) for the Ag NMs at 48 h (Figure 2.3 C).

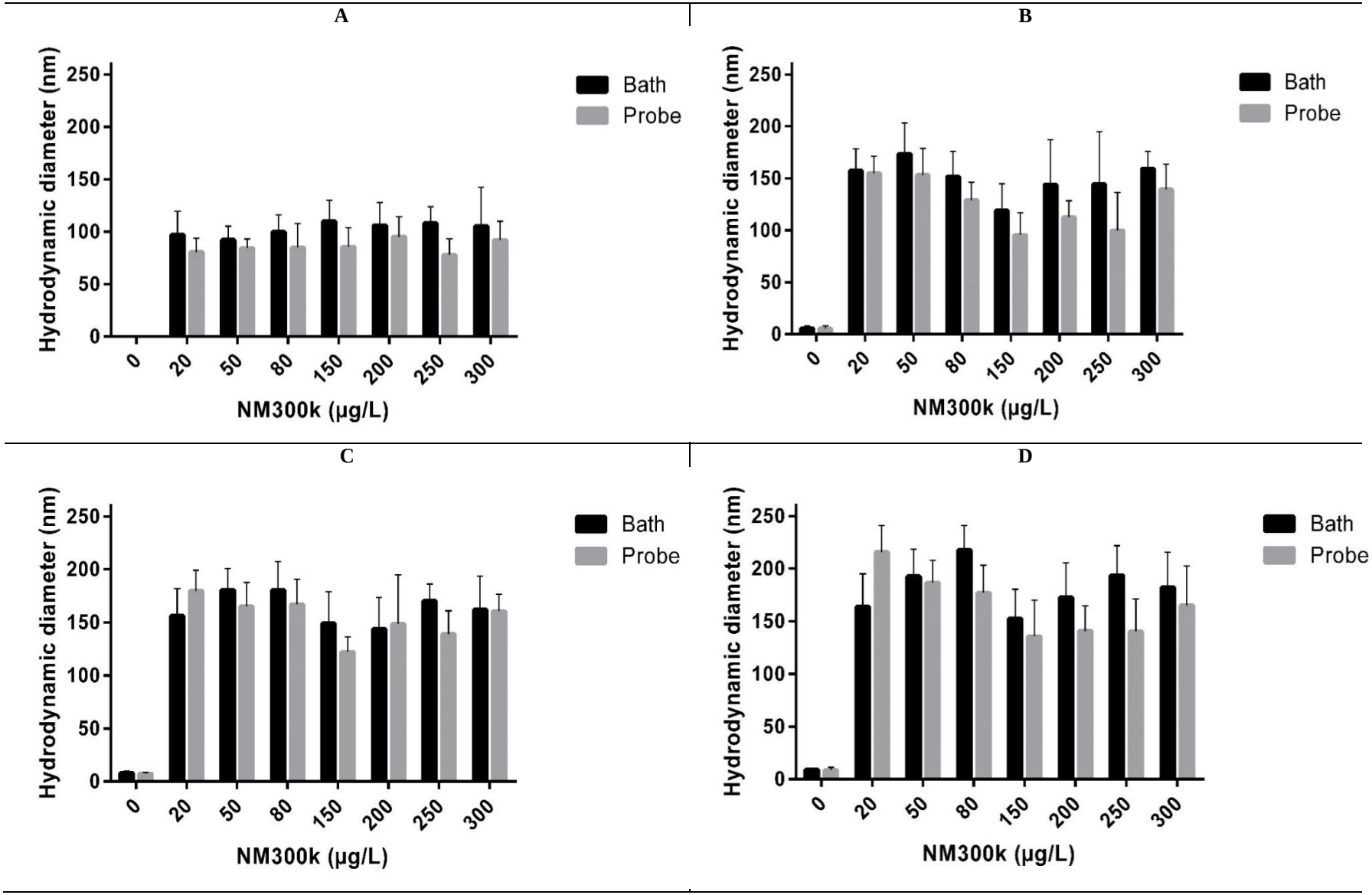


Figure 2.3: Hydrodynamic diameter of Ag NMs obtained by DLS using bath and probe sonication at: 0 h (A), 24 h (B), 48 h (C) and 72 h (D) with different concentrations of Ag NMs suspended in OECD 201 medium, (n=3) (means ± standard deviation).

As illustrated in Figure 2.3 D, at 72 h, the Ag NMs suspensions exhibited a Z-average value across all tested concentrations in the ranges of 152.46–217.63 nm and 135.83–215.73 nm for bath and probe sonication respectively. No significant differences were found between all tested concentrations for Ag NMs particle hydrodynamic diameters using bath sonication at 72 h.

In terms of changes in the particle hydrodynamic diameter of Ag NMs over time (72 h), there was a slight increase in the hydrodynamic diameter of Ag NMs when using bath and probe sonication across all tested concentrations. Ag NMs particle hydrodynamic diameters for all tested concentrations for this time point were between 92.4 and 217.6 nm, and between 78.06 and 215.73 nm for bath and probe sonication respectively. Moreover, at 72 h, there was a significant increase in the particle hydrodynamic diameter of bath-sonicated Ag NMs for 20, 50, 80, 200, 250 and 300 µg Ag/L compared to 0 h (Figure 2.4 A). In contrast, a significant increase in the particles' hydrodynamic diameters for the three lowest tested concentrations (20, 50, 80, 250 and 300 µg Ag/L) of probe-sonicated Ag NMs at 48 and 72 h was observed compared to 0 h (Figure 2.4 B). Based on the results of two-way ANOVA interaction, the hydrodynamic diameter were significantly affected by the interaction of the two factors, time points and sonication type (Table 2.4).

Table 2.4: Results of two-way ANOVA interaction results for hydrodynamic diameter of Ag NMs after exposure to increasing concentrations of Ag NMs for 0, 24, 48 and 72h.

Source	DF	Adj MS	F-Value	*P*-Value
Time points	13	338.1	0.87	0.592
Concentration	13	1670	2.11	0.068
Time points*concentrations	13	3217	1.99	0.025
Sonication type*hydrodynamic diameter	1	11251	6.68	0.011

Generally, the changes in the hydrodynamic diameter of Ag NMs particles suspended in OECD 201 medium using bath and probe sonication over exposure times of 0, 24, 48 and 72 h showed that all tested concentrations (20–300 µg Ag/L) were unstable over time. A time-dependent increase in particle hydrodynamic diameters was also observed (Figure 2.4 A and B).

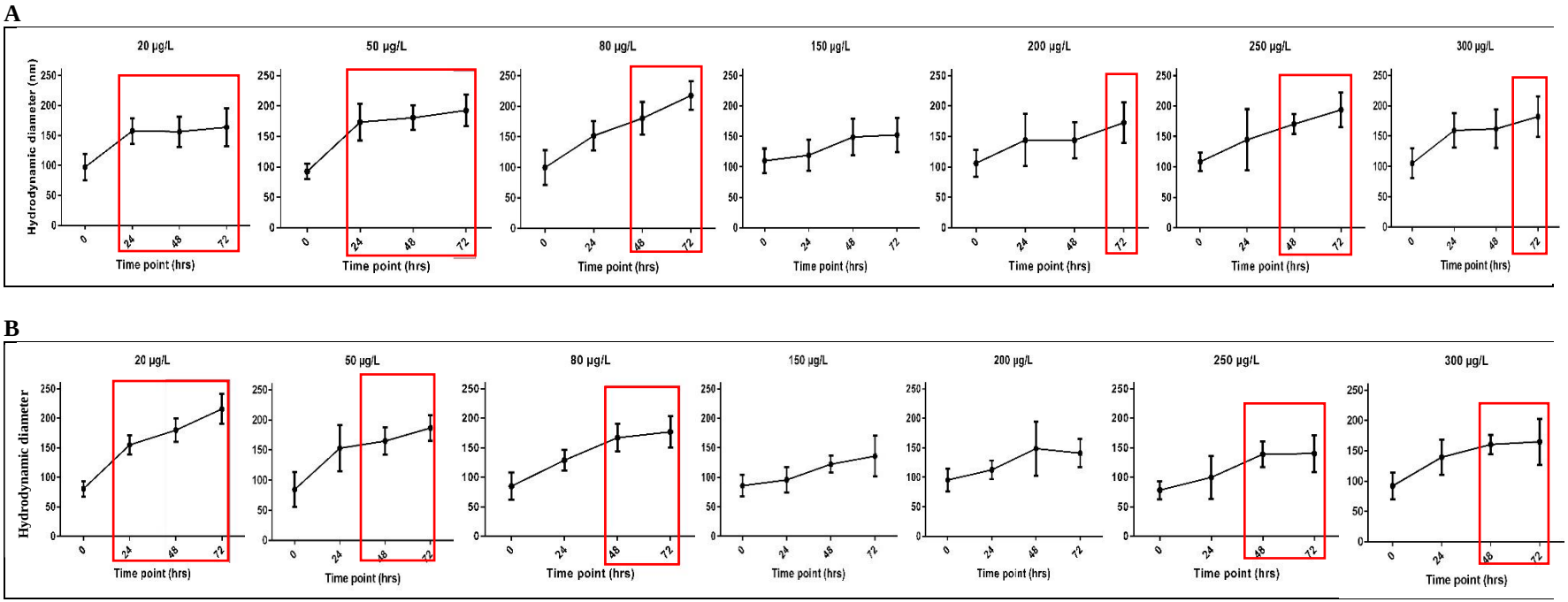


Figure 2.4: Changes in hydrodynamic diameter of Ag NMs particles in OECD 201 medium using bath sonication (A) and probe sonication (B), over experiment time (0, 24, 48 and 72 h). Red squares indicate the significance of tested time points (p<0.05) compared to 0 h

2.3.1.2 Zeta potential

DLS was used to measure the Ag NMs particle zeta potential as well as the hydrodynamic diameter, as discussed (section: 2.3.1.1). Ag NMs was dispersed in OECD 201 medium, at different concentrations (20, 50, 80, 150, 200 and 300 µg Ag/L) of Ag NMs. All zeta potential values obtained were negative. Results showed that the mean zeta potential values across all exposure concentrations immediately after sample preparation, for both types of sonication, were -5.21 to -18.03 mV, and -7.93 to -18.53 mV, respectively, for bath and probe (Figure 2.5 A).

In the control sample (OECD 201 medium only), the average zeta potentials were -3.51 and -5.04 mV for bath- and probe-sonicated samples, respectively. The zeta potential of the lowest concentration (20 µg Ag/L) was significantly different from that of the highest concentration (300 µg/L); these were -5.21 mV and -18.03 mV, respectively. Moreover, in terms of probe sonication, the zeta potential of the lowest concentration (20 µg/L) was significantly different from all other tested concentrations of Ag NMs. Regarding the effect of the sonication type on the zeta potential, there was a significant difference between the different types of sonication (bath and probe), except for the lowest and highest tested concentrations of Ag NMs (20 and 300 µg Ag/L).

After the zeta potential measurements at 0 h, all samples were incubated in the orbital shaker for 72 h, under the same conditions as the toxicity experiment (225 RPM), with a continuous fluorescent light ~120 µmol/m^2.s, at 23° C ± 2° C.

At 24 h, the zeta potentials for all the tested concentrations of Ag NMs were between -8.62 and -13.23 mV, and between -8.36 and -19.3 mV for bath and probe sonication, respectively (Figure 2.5 B). No significant differences were found between zeta potential values for 20, 50 and 80 µg Ag/L using bath sonication. However, significant differences were found between zeta potential values of 20 and 80 µg Ag/L compared to the other tested concentrations (150, 200, 250 and 300 µg Ag/L).

Regarding probe sonication, there were no significant differences between 20 and 80 µg Ag/L, however, these two tested concentrations were found to be significantly different from the 50 µg Ag/L and also from the higher tested concentrations (50, 150, 200, 250 and 300 µg/L). Generally, the results of 150, 200 and 300 µg Ag/L of Ag NMs from bath sonication were significantly different from the results from probe sonication for the Ag NMs zeta potential at 24 h.

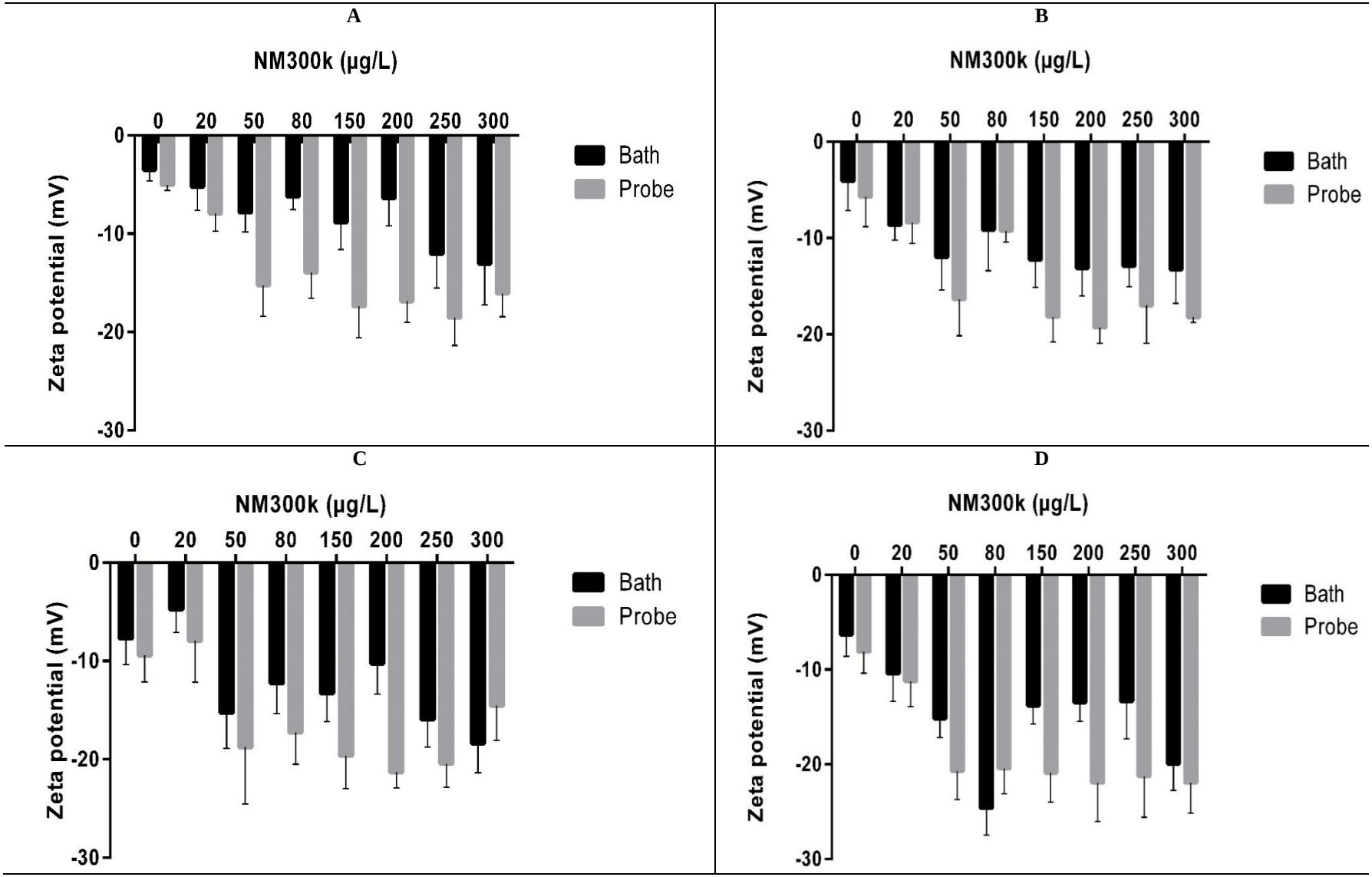


Figure 2.5: Zeta potential of Ag NMs obtained by DLS using bath and probe sonication at: 0 h (A), 24 h (B), 48 h (C) and 72 h (D) with different concentrations of Ag NMs suspended in OECD 201 medium (n=3) (means ± standard deviation).

The Ag NMs zeta potential measurements in the OECD 201 medium at 48 h are displayed in Figure 2.5 C. This shows that the average zeta potential at 48 h was between -4.76 and -18.4 mV, and between -7.92 and -21.3 mV for bath and probe sonication, respectively. Considering the Ag NMs zeta potential using bath sonication, there was a significant difference between the lowest tested concentration (20 µg Ag/L) and the other tested concentrations. In contrast, by using probe sonication, the lowest tested concentration (20 µg Ag/L) was significantly different from the other tested concentrations of 50, 80 150, 200, 250 and 300 µg Ag/L.

In addition, bath sonication for Ag NMs was found to be significantly different from probe sonication for some of the tested concentrations (150 and 200 µg Ag/L) in terms of the zeta potential at 48 h.

The results of the Ag NMs zeta potential measurements after 72 h in the OECD 201 medium are displayed in Figure 2.5 D, which shows the average zeta potential to be between -10.42 and -24.6 mV, and between -11.24 and -21.93 mV for bath and probe sonication, respectively. There was a significant difference between the zeta potential at 80 and 300 µg Ag/L of Ag NMs using bath sonication and the rest of the tested concentrations; the lowest tested concentration was found to be significantly different from the highest. In contrast, at 72 h the lowest tested concentration was significantly different from the rest of the tested concentrations for the Ag NMs zeta potential using probe sonication. Furthermore, the zeta potential of bath-sonicated Ag NMs was found to be significantly different from that of probe-sonicated Ag NMs at 72 h.

Overall, in terms of sonication type, bath sonication was significantly different from probe sonication for the zeta potential of Ag NMs across all time points. Based on the results of two-way ANOVA interaction zeta potential were significantly affected by the interaction of the two factors, time points and sonication type (Table 2.5).

Table 2.5: Results of two-way ANOVA interaction results for zeta potential of Ag NMs after exposure to increasing concentrations of Ag NMs for 0, 24, 48 and 72h.

Source	DF	Adj MS	F-Value	*P*-Value
Time points	3	272.1	30.19	0.001
Concentration	13	190.846	21.17	0.354
Time points*Concentration	39	18.087	2.01	0.002
Sonication type*zeta potential	1	892.358	44	0.001

The zeta potential values over the experiment for all the tested concentrations of Ag NMs were from -4.76 to -24.6 mV, and from -7.92 to -21.93 mV for bath and probe sonication respectively (Figure 2.6). Over time, the zeta potential of Ag NMs decreased for both bath and probe sonication across all tested concentrations.

Furthermore, as illustrated in Figure 2.6 A, there was a significant decrease in the zeta potential of the bath-sonicated Ag NMs for 50 and 300 µg Ag/L and 200 µg Ag/L probe sonicated after 48 and 72 h, respectively, compared to 0 h. In addition, for the 150 µg/L concentration, there was a decrease in the zeta potential, from 24 h onward, compared to 0 h. Generally, there were a significant decrease in the zeta potential for most of the tested concentrations of bath sonicated Ag NMs except 250 µg/L at 72 h compared to 0 h. In contrast, when using probe sonication, a significant decrease in the zeta potential of Ag NMs was observed for most of the tested concentrations at 72 h (Figure 2.6 B).

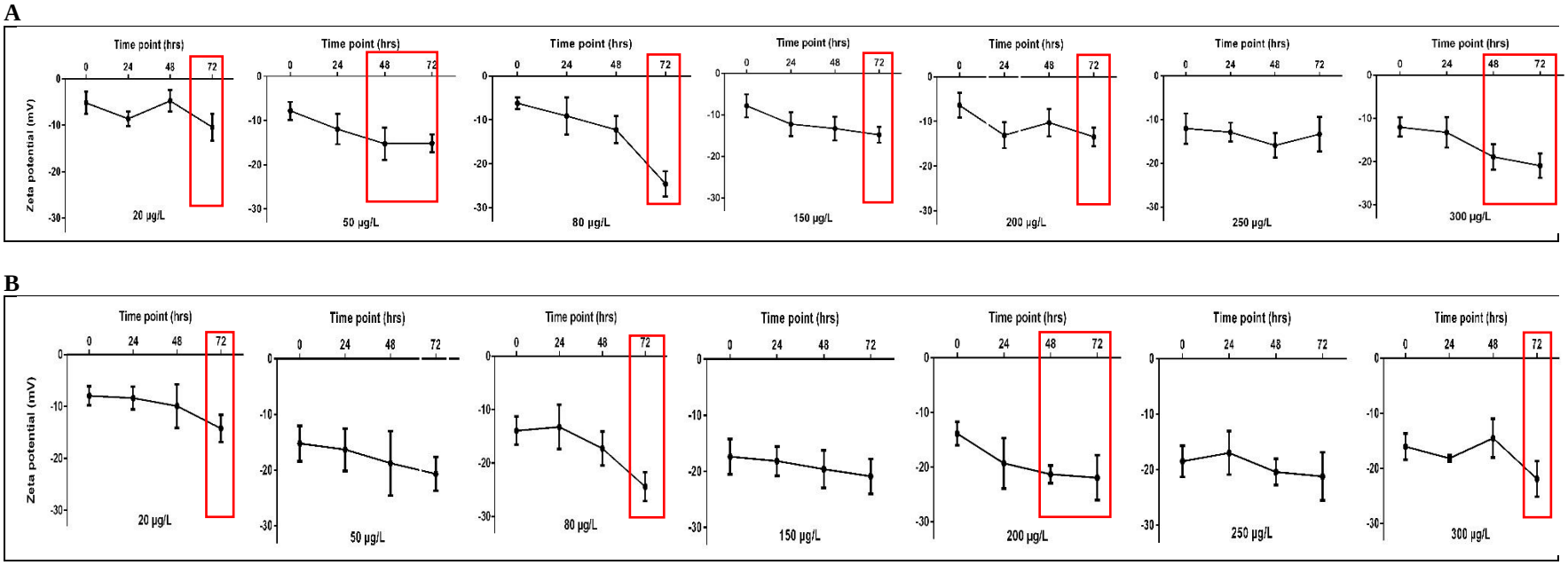


Figure 2.6: Changes in zeta potential of Ag NMs in OECD 201 medium using bath sonication (A) and probe sonication (B), over experiment time points (0, 24, 48 and 72 h). Red squares indicate the significance of tested time points (p<0.05) compared to 0 h.

2.3.2 *TEM*

A visual examination of the primary NM and agglomerate sizes, as well as shape and tube structure of the tested NMs, was determined by TEM. TEM images illustrated the size and morphology of NMs suspended in the OECD 201 medium at 0 h (immediately after sample preparation). Imaging by TEM was only possible in the stock suspensions (10 mg Ag/L) prepared via bath and probe sonication using two different procedures for TEM sample preparation: the drop cast and plunge freezing procedures. Dispersion of NMs was assessed using a TEM (FEI Tecnai F20 FEG-TEM operated at 200 kV) at the University of Leeds, UK.

2.3.2.1 Drop casting

2.3.2.1.1 Bath- and probe-sonicated Ag NMs

Images of Ag NMs for samples prepared using drop casting are displayed in Figures 2.7 and 2.8 for bath and probe sonication, respectively. The images for the Ag NMs suspensions, bath and probe sonicated, showed single NMs as well as agglomerated structures. As measured by TEM, the average diameter of the Ag NMs stock suspension (10 mg Ag/L) was <20 nm for both bath- and probe-sonicated Ag NMs. According to the TEM images, the morphology of the Ag NMs was close to spherical (mainly euhedral) (Figure 2.7 and 2.8).

TEM was used to validate the stated primary particle size of the Ag NMs (Klein et al. 2011), and results obtained indicate good concordance with other published studies which investigated the size and morphology of Ag NMs in different media using TEM (Kermanizadeh et al. 2013; Losasso et al. 2014; Donnellan et al. 2016; Kleiven et al. 2018). In terms of the agglomeration status, both types of sonication resulted in a degree of agglomeration. However, after bath sonication, the Ag NMs appear to have a higher degree of agglomeration (Figure 2.7) compared to the samples prepared by probe sonication, which showed looser agglomeration, that is, the probe sonication seems to result in better dispersion of NMs (Figure 2.8).

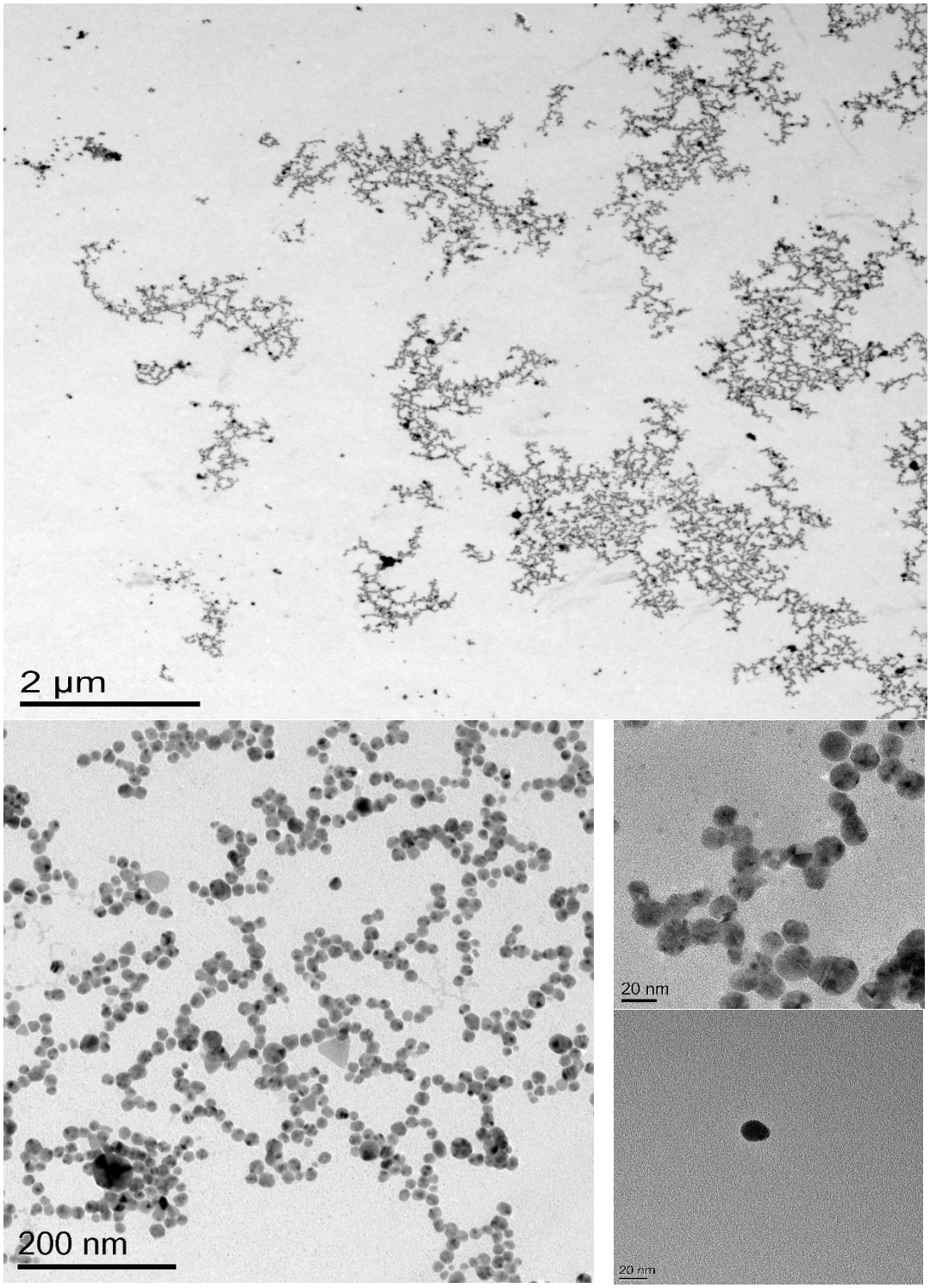


Figure 2.7: TEM images of Ag NMs after bath sonication in OECD 201 medium containing 10 mg/L at 0 h. Ag NMs suspensions were dried overnight on a carbon film-coated Cu grid at room temperature. Scale bars = 2 µm (top), 200 nm (bottom left), 20 nm (middle right) and 20 nm (bottom right).

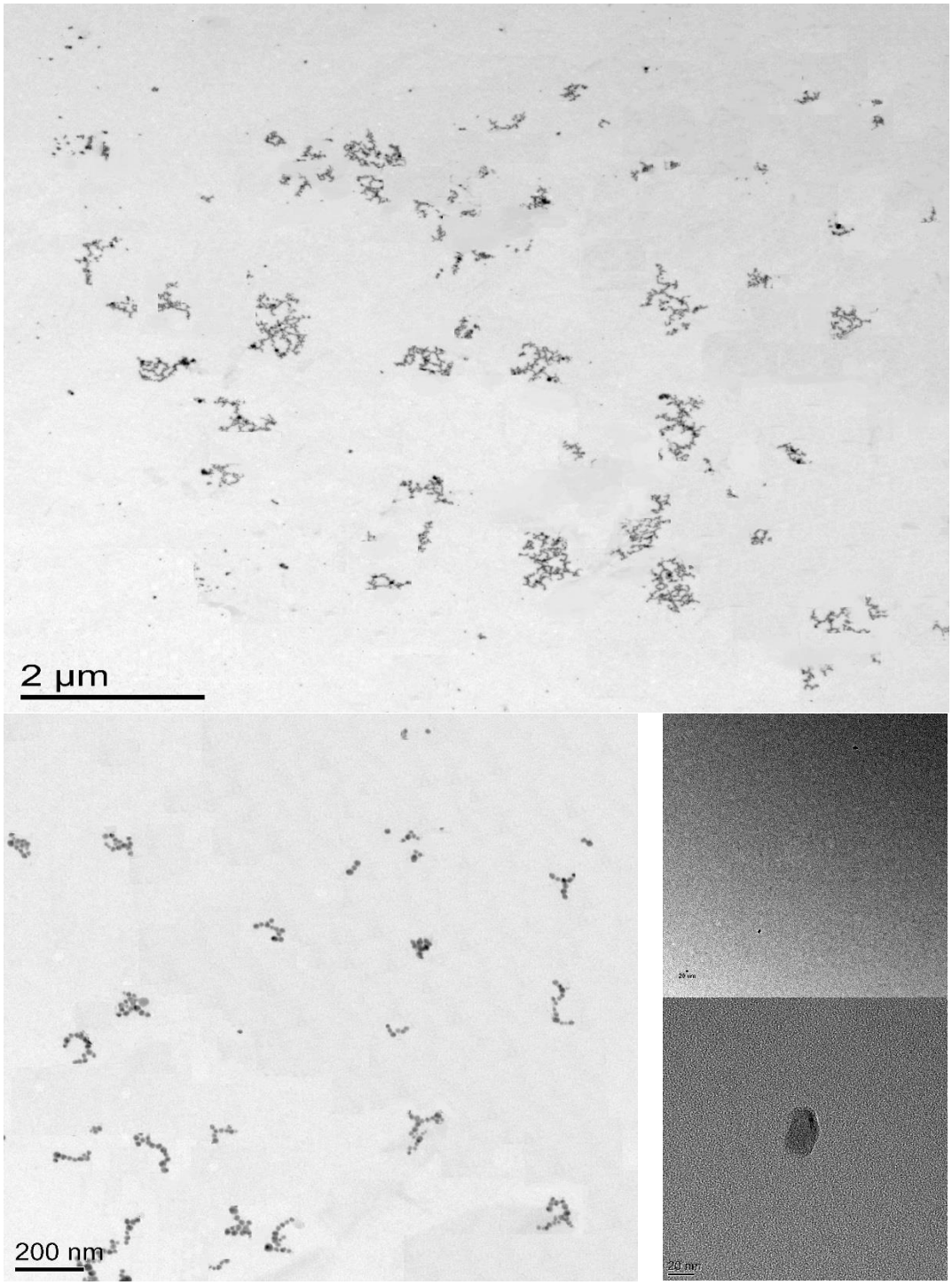


Figure 2.8: TEM images of Ag NMs after probe sonication in OECD 201 medium containing 10 mg Ag/L at 0 h. Ag NMs suspensions were dried overnight on carbon film-coated Cu grid at room temperature. Scale bars = 2 µm (top), 200 nm (bottom left), 20 nm (middle right) and 20 nm (bottom right).

2.3.2.1.2 Ag NMs – EDX measurements

The composition of Ag NMs was analysed using EDX in the OECD 201 medium. The EDX approach is suitable for major and minor elements. From each sample, at least two places were chosen for EDX analysis: one as a background for the Cu grid (away from NM) (e.g. Figures 2.9 A and 2.10 A) and the second on the NM itself (e.g. Figures 2.15 B and 2.16 B). The EDX spectra show no signs of any other elements except Cu, as the grid is made from Cu (EDX spectrum A), and in the second EDX spectra, the elemental peaks which signal the presence of Ag are very clear (EDX spectrum B). Furthermore, the EDX spectra from the Ag NMs indicate that there was a little sulphur (S) and silicon (Si) present, which came from the contents of the medium.

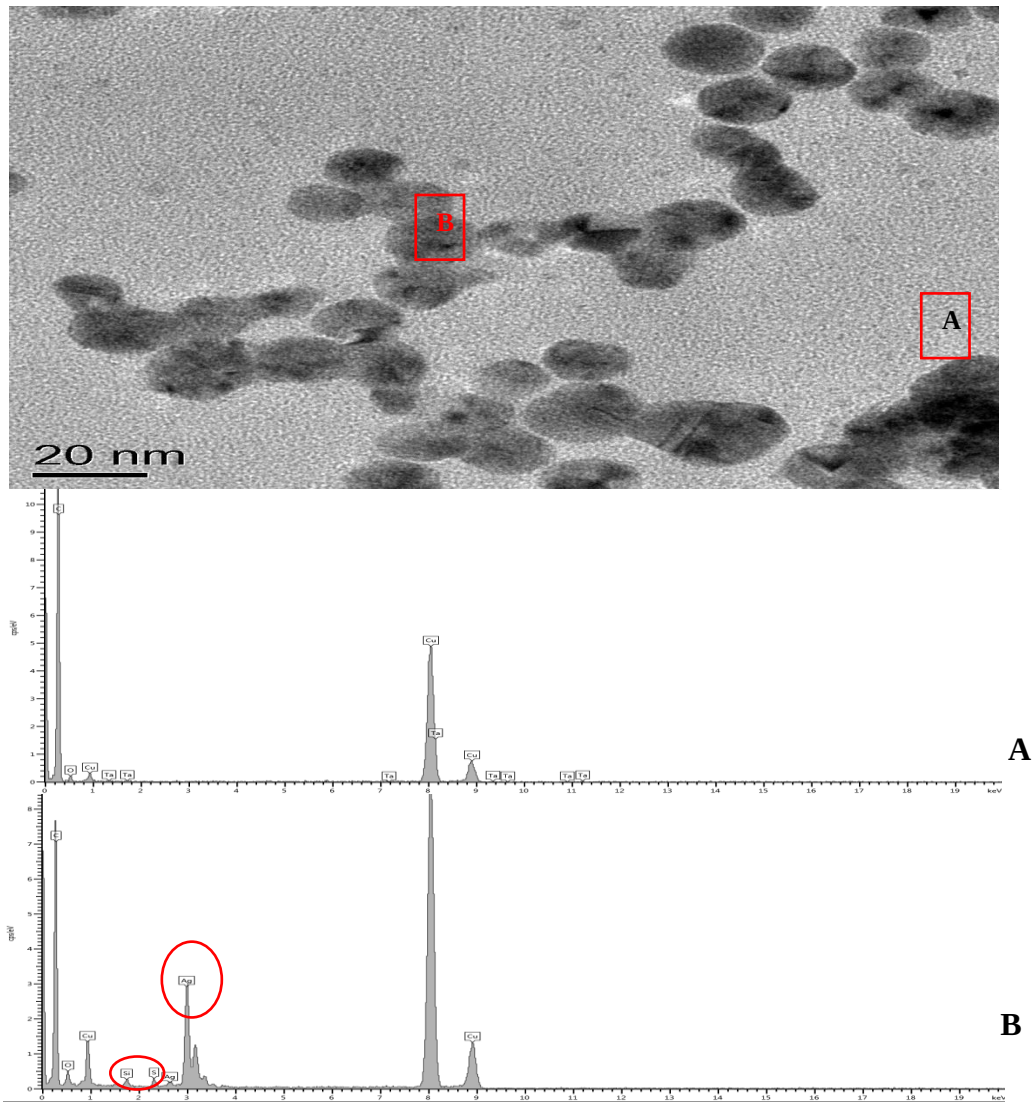


Figure 2.9: Energy-dispersive X-ray (EDX) spectra for the grid only (A) and for the NMs (B). The spectra show Ag peaks (B) for Ag NMs (10 mg/L) after bath sonication in OECD 201 medium. Red outlines indicate areas analysed for Ag NM signals and the labels for Ag NM, Si and S peaks.

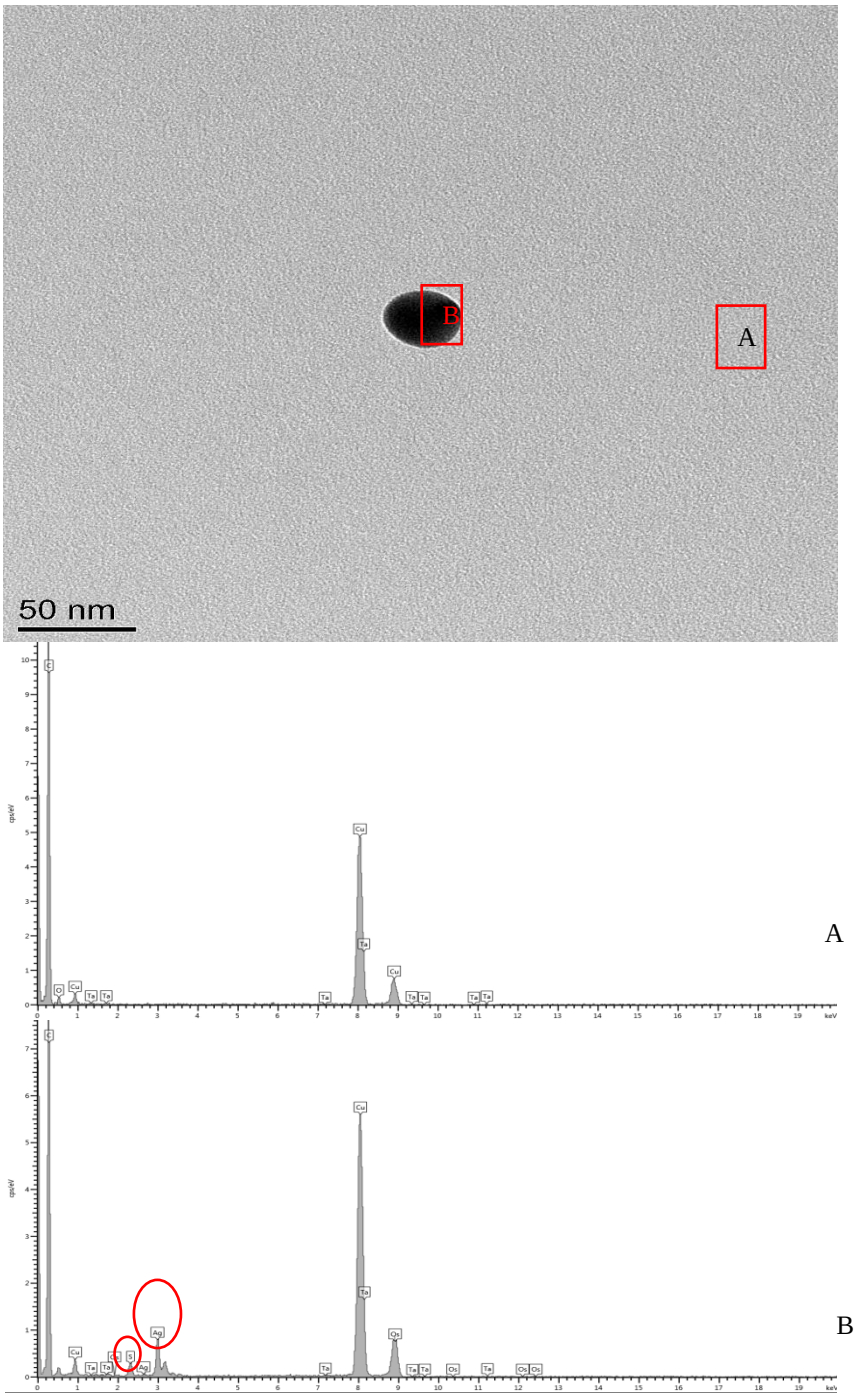


Figure 2.10: Energy-dispersive X-ray (EDX) spectra for the grid only in red square (A) and for the Ag NMs in red square (B). The spectra show Ag peaks (B) for Ag NMs (10 mg/L) after probe sonication in OECD 201 medium. Red outlines indicate areas analysed for Ag NMs signal and the labels for Ag NM and S peaks.

2.3.2.2 Plunge freezing

2.3.2.2.1 Bath- and probe-sonicated Ag NMs

Figures 2.11 and 2.12 display images from the TEM samples prepared using plunge freezing in liquid ethane immediately after the NM sample preparation. These TEM images indicate good dispersion overall, with probe-sonicated samples indicating a better dispersion compared to bath-sonicated samples.

Bath-sonicated samples showed agglomerates of a few NMs up to approximately 80 nm, whereas probe-sonicated samples showed little agglomeration. Moreover, a number of individual particles and very loosely associated NMs could be seen as well for both bath- and probe-sonicated samples (Figures 2.11 and 2.12).

Differences between sampling protocols, concentrations and time points tested, combined with the possibility of drying artefacts (Tauer 2004; Domingos et al. 2009), could account for differences in the agglomeration status of NMs observed in different studies.

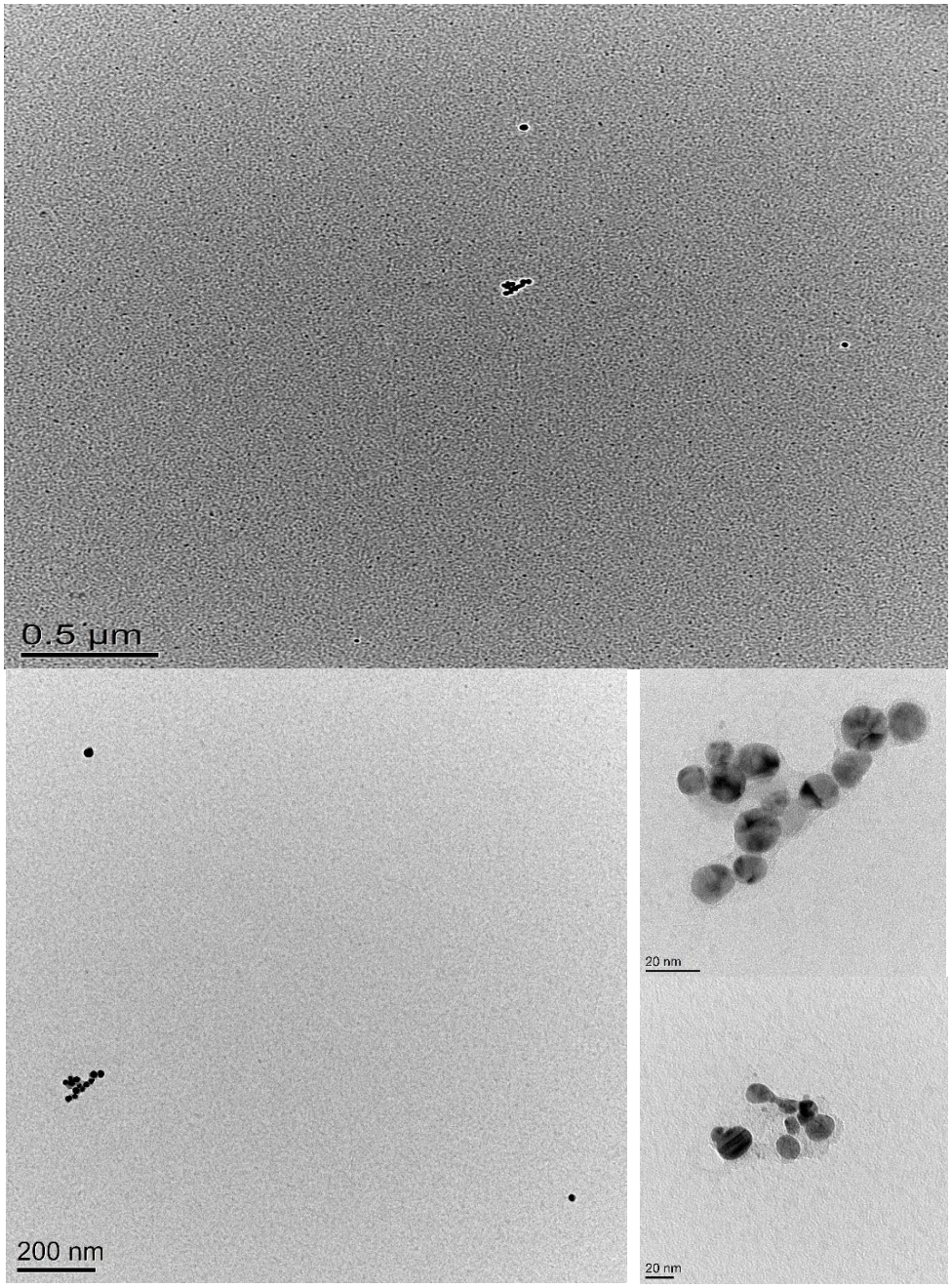


Figure 2.11: TEM images of Ag NMs after bath sonication in OECD 201 medium containing 10 mg Ag/L at 0 h. Ag NMs suspensions were plunge frozen on carbon film-coated Cu grid at room temperature. Scale bars = 0.5 µm (top), 200 nm (bottom left), 20 nm (middle right) and 20 nm (bottom right).

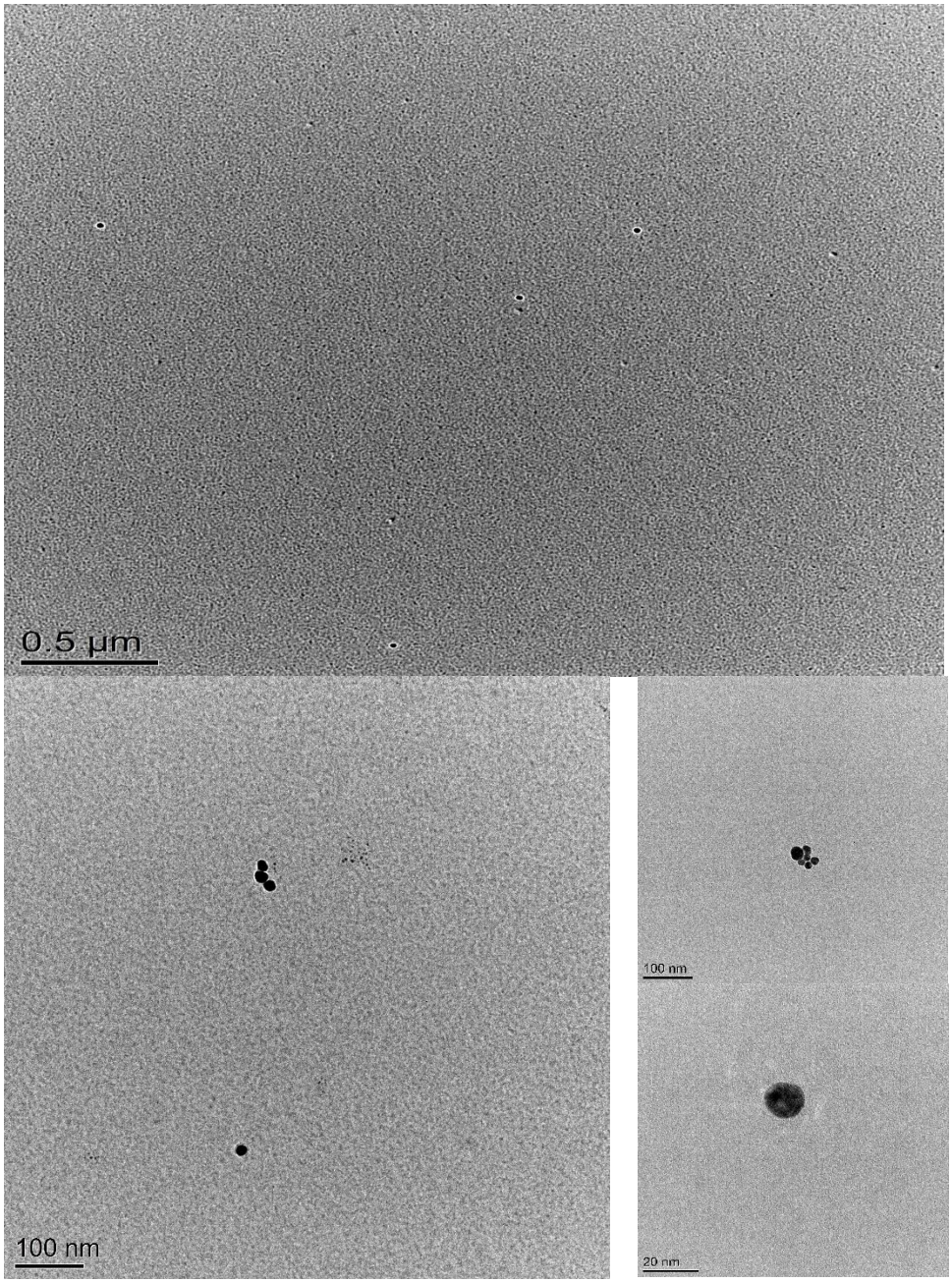


Figure 2.12: TEM images of Ag NMs after probe sonication in OECD 201 medium containing 10 mg Ag/L at 0 h. Ag NMs suspensions were plunge frozen on carbon film-coated Cu grid at room temperature. Scale bars = 0.5 µm (top), 100 nm (bottom left), 20 nm (middle right) and 20 nm (bottom right).

2.3.2.3 Drop casting

2.3.2.3.1 Bath- and probe-sonicated MWCNTs

As for Ag NMs, MWCNTs (NM400) were characterised in the stock suspension (10 mg/L) and in the OECD 201 medium using both types of sonication (bath and probe). The dispersion of MWCNTs in the OECD 201 medium was followed by the drop cast method when preparing the TEM samples. Results are displayed in Figures 2.13 and 2.14, for bath and probe sonication, respectively. Two additional samples were prepared using plunge freezing for TEM analysis, and assessed using a TEM model FEI Tecnai F20 FEG-TEM operated at 200 kV.

TEM was used to assess MWCNT (NM400) agglomeration status, and the general morphology as well as the presence of contaminants compared with results obtained in Milli-Q water suspensions (Rasmussen et al. 2014). Results obtained indicated good concordance with other published studies which investigated the size and morphology of MWCNTs in different media using TEM (Kermanizadeh et al. 2013; Vietti et al. 2013; Cao et al. 2014; Öner et al. 2016; Bermejo-Nogales et al. 2017; Farkas and Booth 2017; Ghosh et al. 2018). In terms of agglomeration status, bath-sonicated MWCNTs showed some agglomeration (Figure 2.13). In the case of probe sonication, it can be noticed that there is no large agglomeration and the MWCNTs seem to be well dispersed (Figure 2.14). MWCNTs appeared to consist of entangled, irregular, and bent nanotubes, and it was difficult to measure the length of the nanotubes by using TEM. Moreover, different layers (~10–20 layers) were seen on the MWCNT wall.

For both sonication approaches, the MWCNTs appeared to contain a fraction of micrometre-sized particles or crystals. These were previously identified as corundum (aluminium oxide) crystals used as support material for NM400 (Rasmussen et al. 2014).

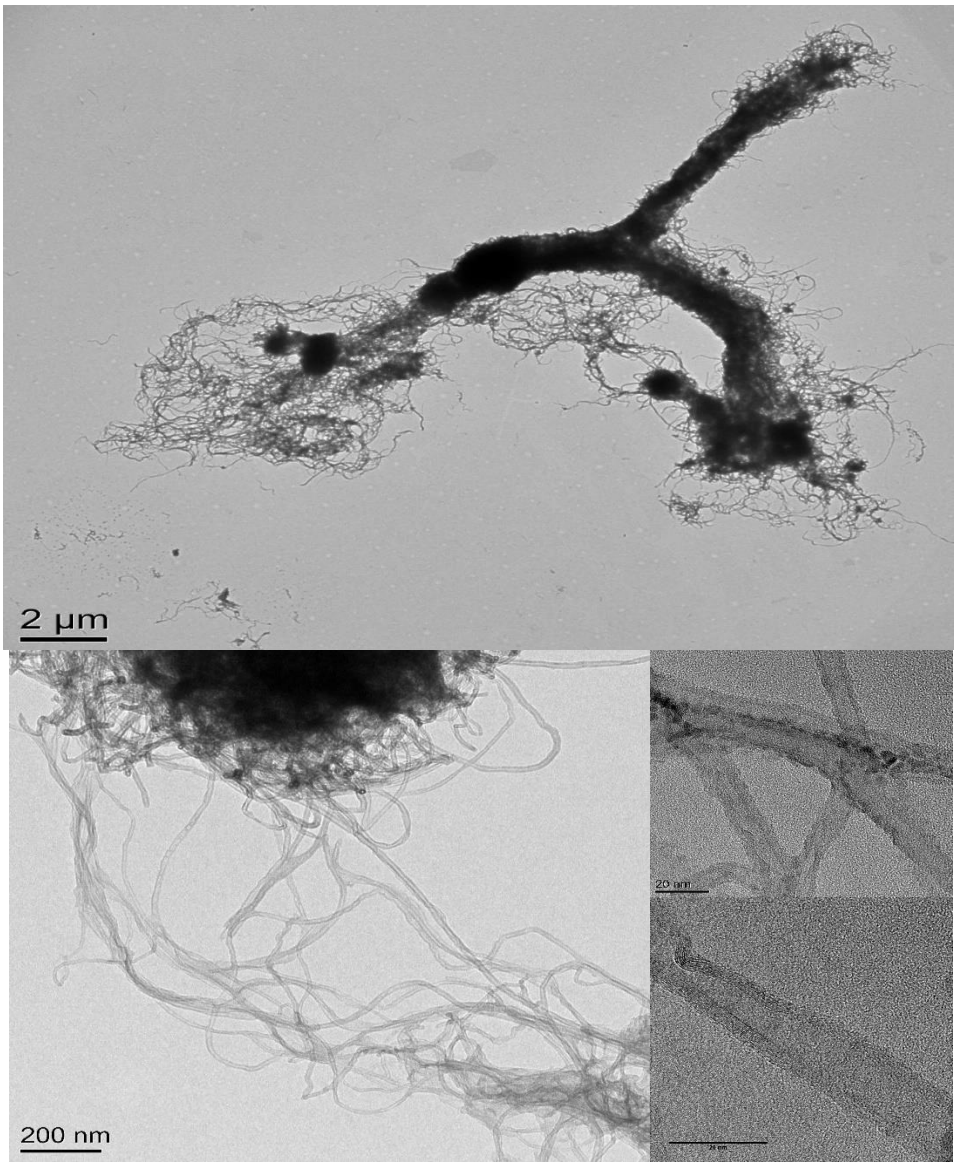


Figure 2.13: TEM images of MWCNTs after bath sonication in OECD 201 medium containing 10 mg/L of MWCNTs at 0 h. MWCNT suspensions were dried overnight on carbon film-coated Cu grid at room temperature. Scale bars = 2 µm (top), 200 nm (bottom left), 20 nm (middle right) and 20 nm (bottom right).

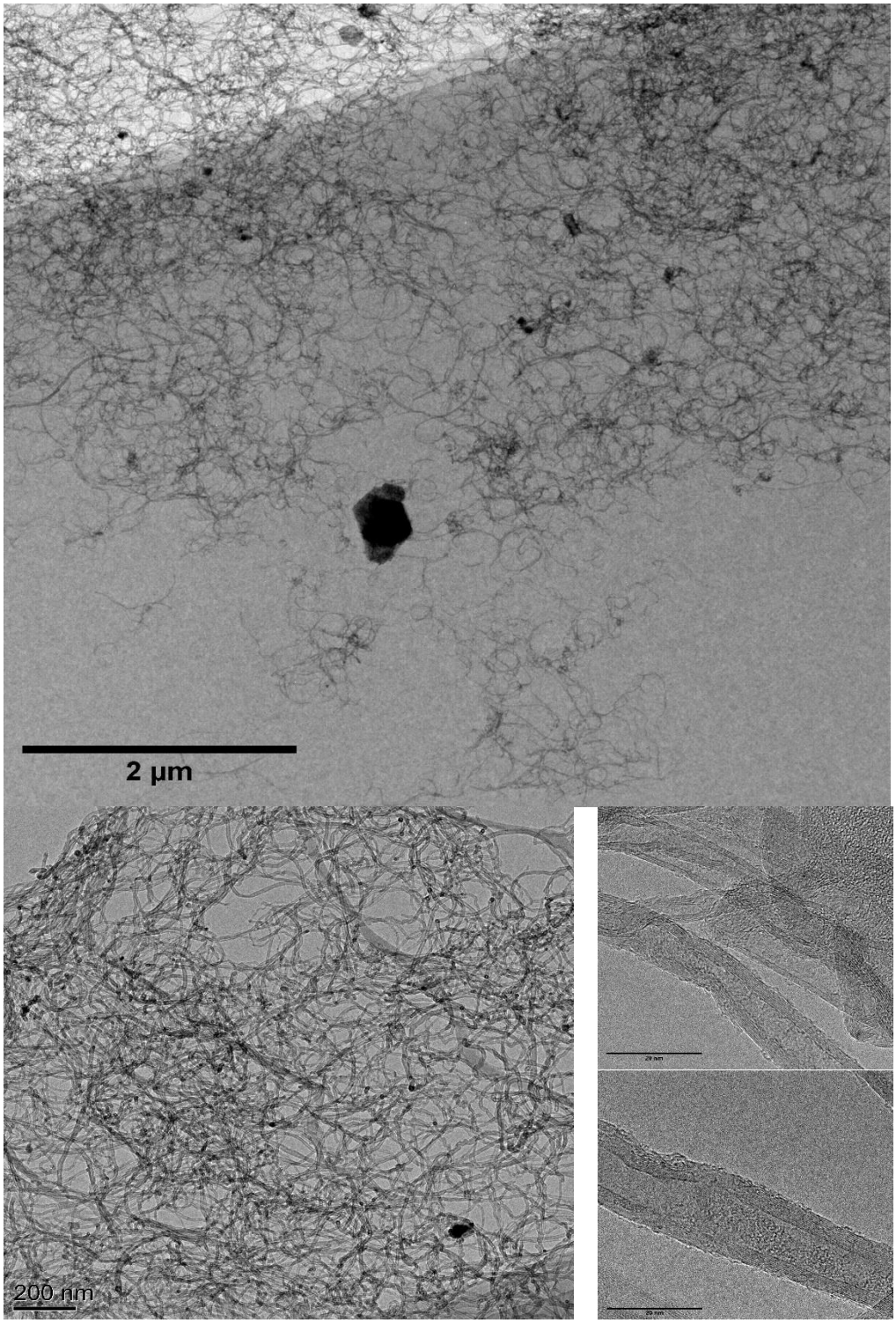


Figure 2.14: TEM images of MWCNTs after probe sonication in OECD 201 medium containing 10 mg/L of MWCNTs at 0 h. MWCNT suspensions were dried overnight on carbon film-coated Cu grid at room temperature. Scale bars = 2 µm (top), 200 nm (bottom left), 20 nm (middle right) and 20 nm (bottom right).

2.3.2.3.2 MWCNT EDX analyses

EDX analysis was performed on the MWCNT samples in order to confirm the possible presence of metal impurities associated with MWCNTs. The EDX spectra indicated that there were some impurities related to MWCNTs such as iron (Fe) and some OECD 201 medium contents, such as silicon (Si), sodium (Na) and sulphur (S) (Figures 2.15 and 2.16), which was found to be in close agreement with other published studies (Hougaard et al. 2013; Vietti et al. 2013; Rasmussen et al. 2014; Öner et al. 2016). The red square (A) indicates the point of EDX analysis as a background measurement on the grid and the EDX spectra (A) from the grid indicated that there were Cu peaks which came from the grid, and S, Si and Na peaks which came from the contents of the medium. In contrast, the red square (B) indicates the point of EDX analysis on MWCNT agglomeration on the grid, and the EDX spectra (B) from the grid indicates that there were Fe peaks, which indicates impurities related to MWCNTs, and Si peaks, which also come from the contents of the medium.

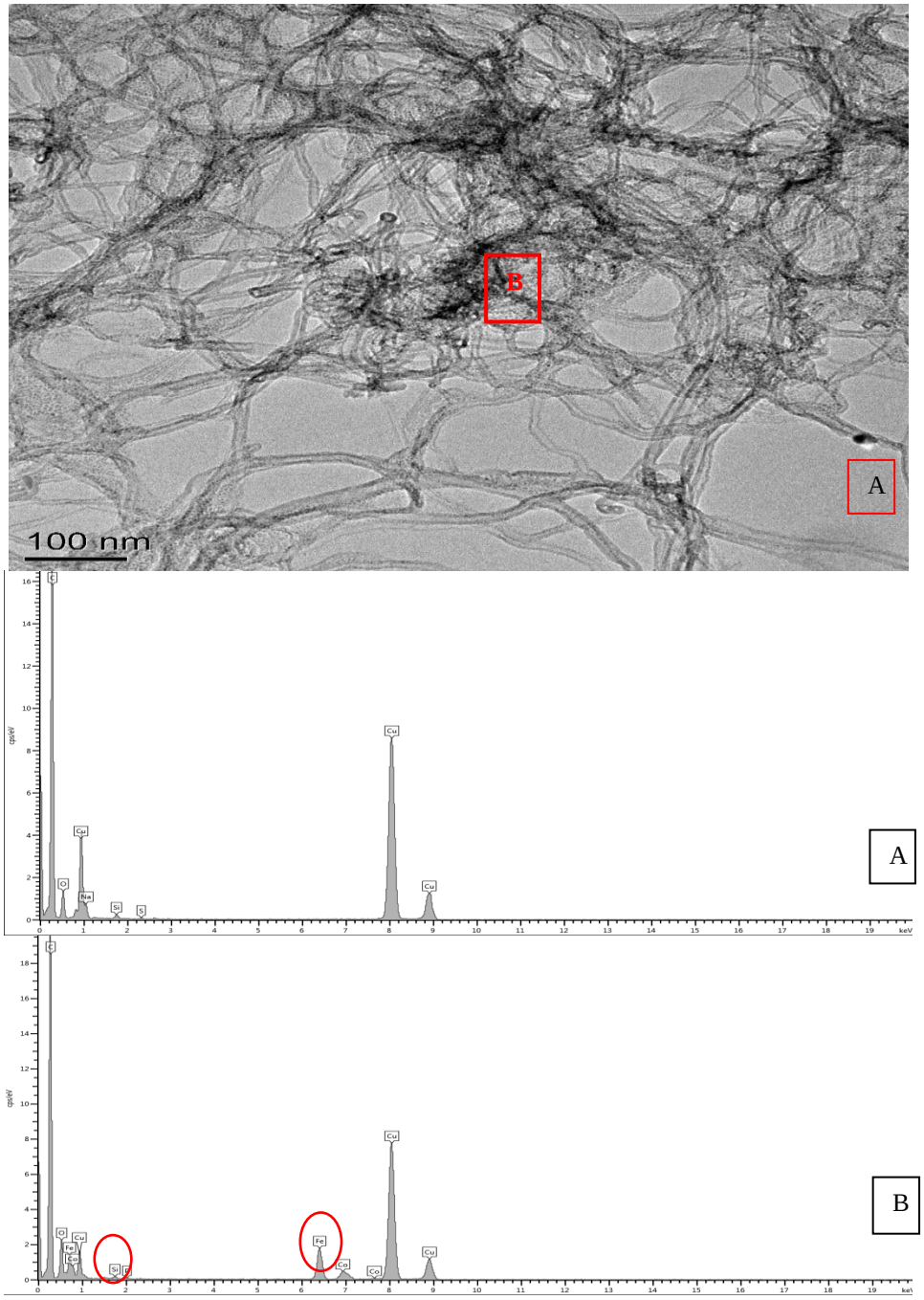


Figure 2.15: Energy-dispersive X-ray (EDX) spectra for the grid only in red square (A) and for MWCNTs (10 mg/L) after bath sonication (red square B) in OECD 201 medium. Red circles in spectra B indicate some impurities related to MWCNTs, such as iron (Fe), and some medium content such as sulphur (S).

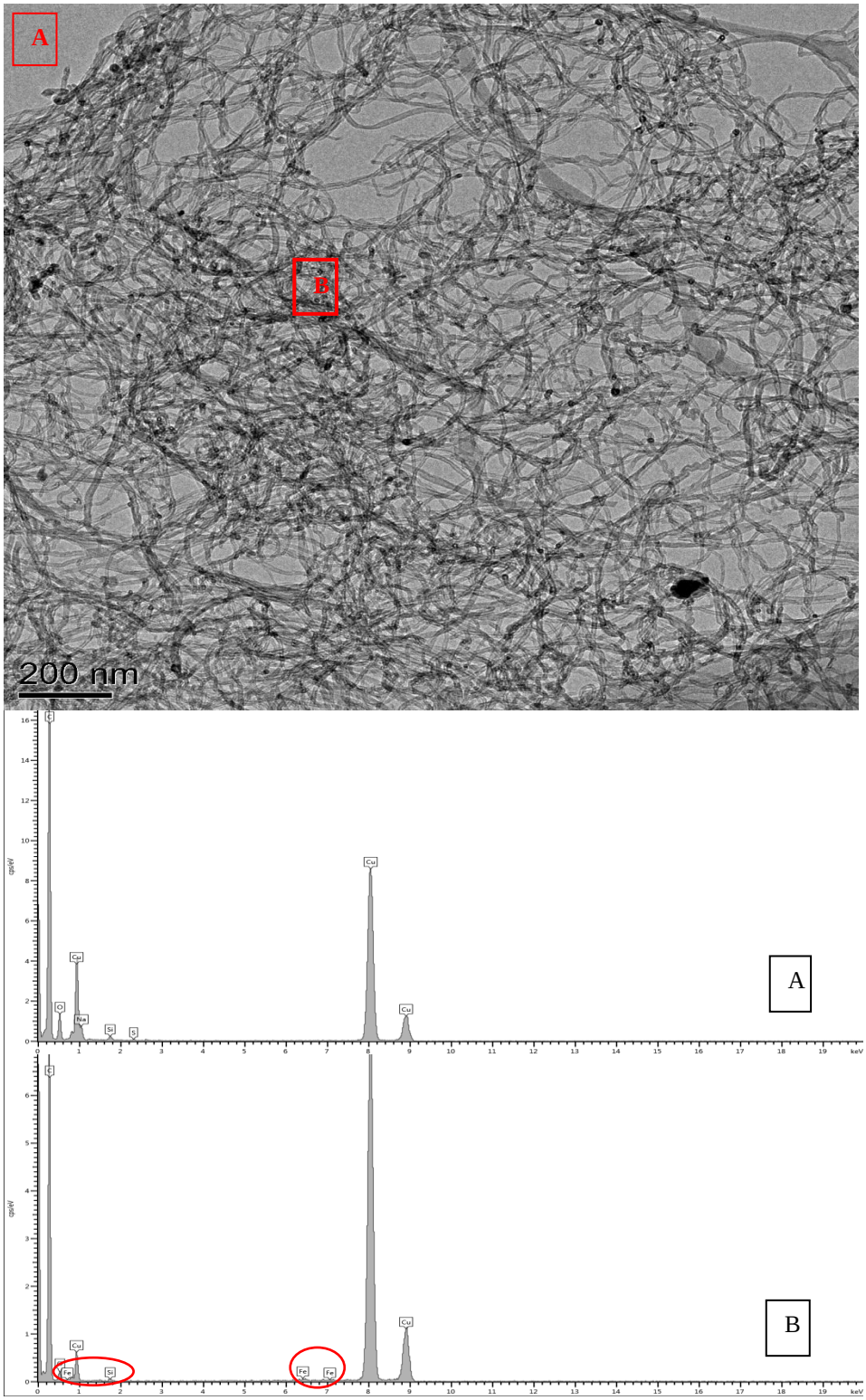


Figure 2.16: Energy-dispersive X-ray (EDX) spectra for the grid only in red square (A) and for MWCNTs (10 mg/L) after probe sonication (red square B) in OECD 201 medium. Red circles in spectra B indicate some impurities related to MWCNTs, such as iron (Fe), and some medium content, such as silicon (Si).

2.3.2.4 Plunge freezing

2.3.2.4.1 Bath- and probe-sonicated MWCNTs

The bath-sonicated MWCNTs appeared agglomerated in the OECD 201 medium (Figure 2.17), but in contrast, probe-sonicated samples seemed better dispersed (Figure 2.18). The MWCNTs appeared to consist of entangled, irregular, and bent nanotubes, and it was difficult to measure their length. However, in the case of probe sonication, some of the individual nanotubes were clear, and their diameters could be measured (<1 µm) (Figure 2.18, middle right). NM400 appeared to contain a fraction of µm-sized particles, which were sometimes identified as fully euhedral graphite crystals such as corundum crystals (Aluminium Oxide – Al_2O_3) used as a catalyst support material (Rasmussen et al. 2014).

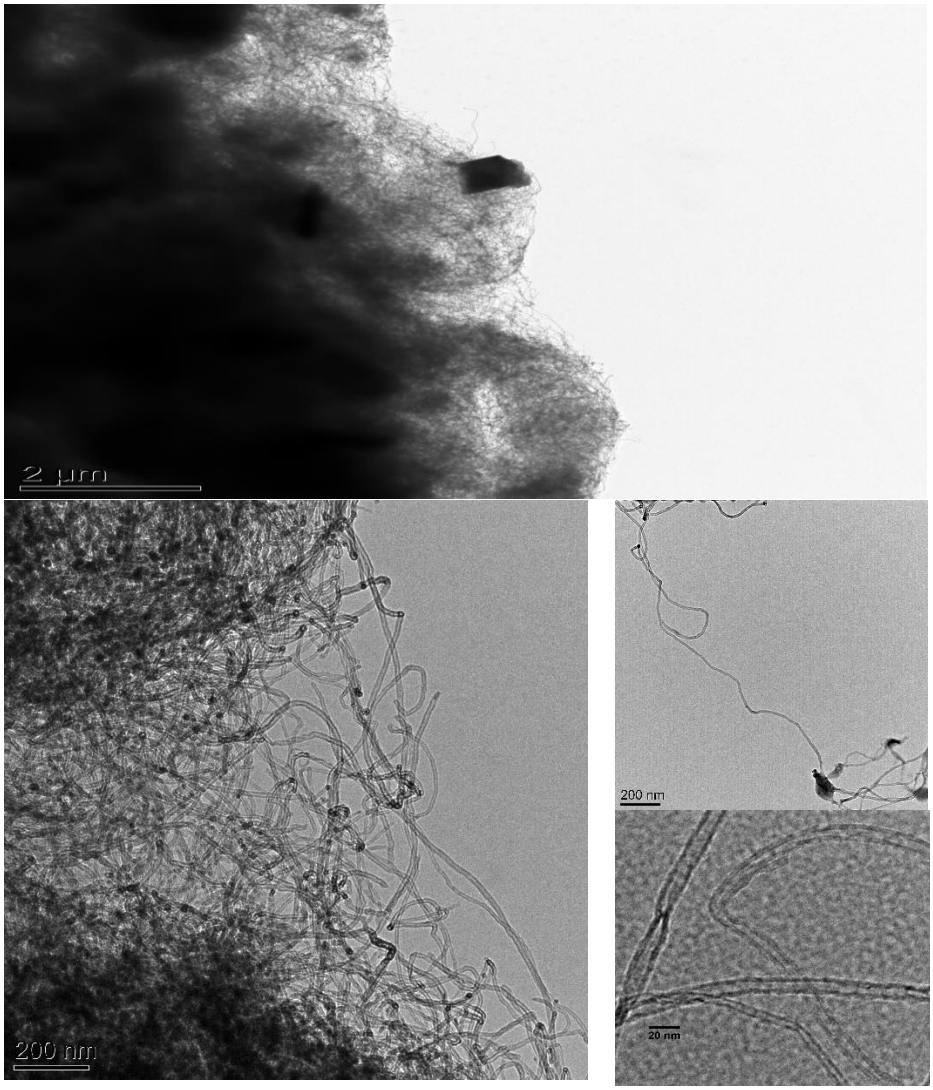


Figure 2.17: TEM images of MWCNTs after bath sonication in OECD 201 medium containing 10 mg/L of MWCNTs at 0 h. MWCNT suspensions were plunge frozen on carbon film-coated Cu grid. Scale bars = 2 µm (top), 200 nm (bottom left), 200 nm (middle right) and 20 nm (bottom right).

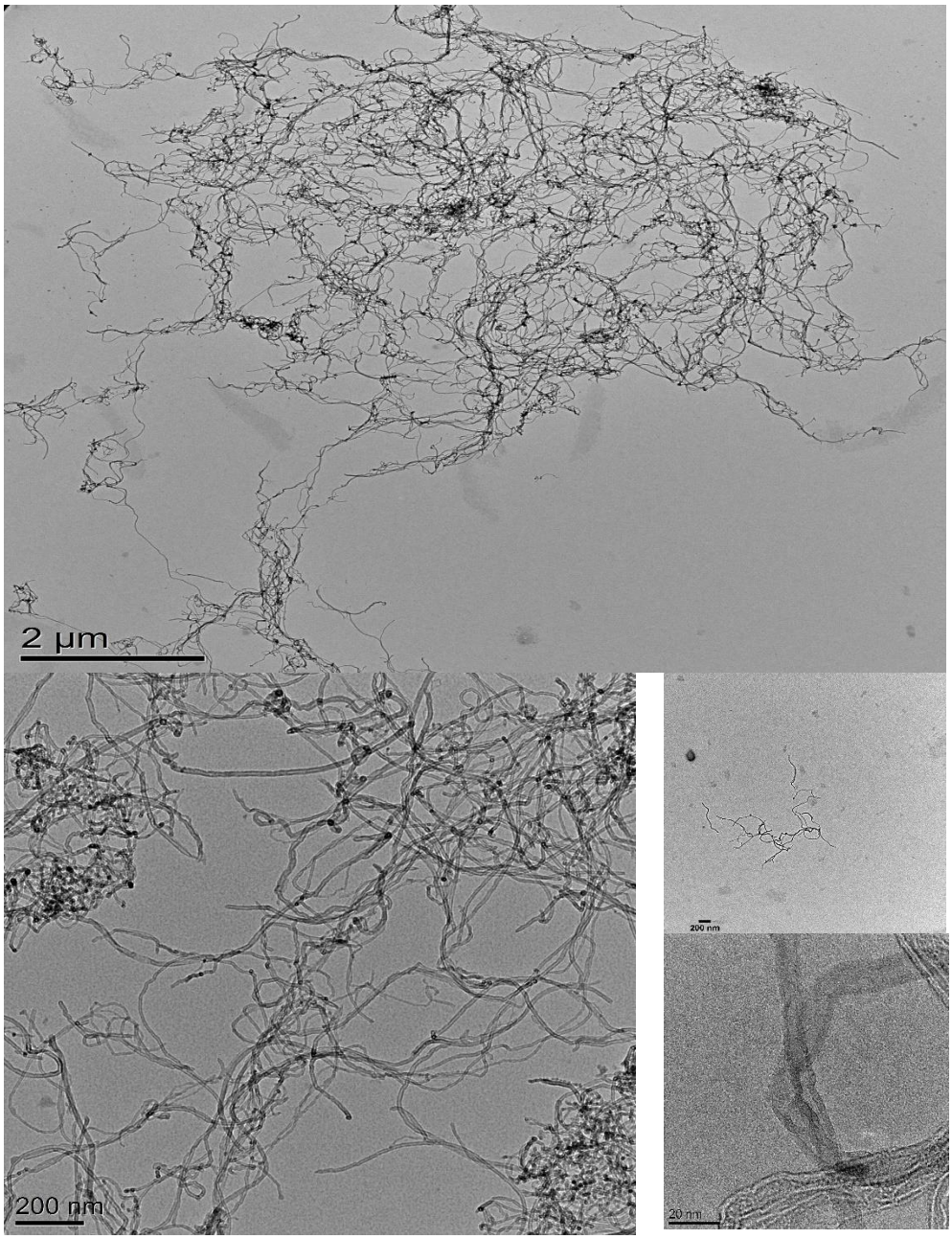


Figure 2.18: TEM images of MWCNTs after probe sonication in OECD medium containing 10 mg/L of MWCNTs at 0 h. MWCNT suspensions were plunge frozen on carbon film-coated Cu grid. Scale bars = 2 µm (top), 200 nm (bottom left), 200 nm (middle right) and 20 nm (bottom right).

2.3.3 *Visual examination*

This study was carried out to determine the stability of MWCNTs in Milli-Q water as well as in OECD 201 medium. The MWCNTs (100 mg/L) as a stock concentration were bath sonicated (for 2 × 8 min) or probe sonicated in ice bath (for 14.11 min) in Milli-Q water and in OECD 201 medium. A volume of 20 mL of this suspension was immediately transferred into glass vials (Figures 2.19 and 2.20) for a visual examination of the stability of particulate MWCNT (NM400) in Milli-Q water and in OECD 201 medium.

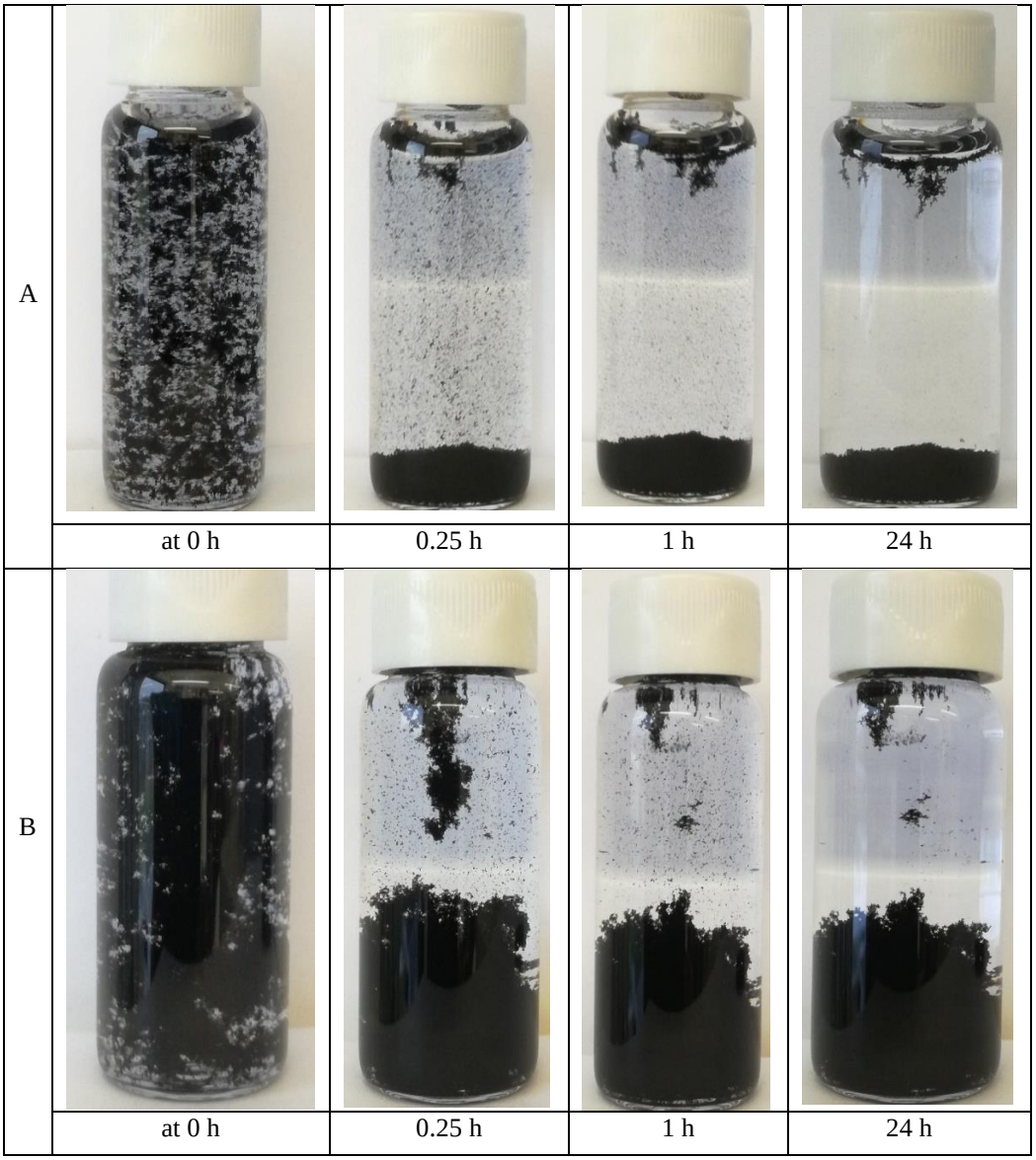


Figure 2.19: Visual examination of the stability of MWCNTs (NM400) in Milli-Q water after bath sonication for 2 × 8 min (A) and after probe sonication in ice bath for 14.11 min (B) at a concentration of 100 mg/L.

A				
	at 0 h	0.25 h	1 h	24 h
B				
	at 0 h	0.25 h	1 h	24 h

Figure 2.20: Visual examination of the stability of MWCNTs (NM400) in OECD 201 medium after bath sonication for 2 × 8 min (A) and after probe sonication in ice bath for 14.11 min (B) at a concentration of 100 mg/L.

It is clear that MWCNTs are not very easily suspended in Milli-Q water, or in OECD 201 medium, by using bath sonication. Following the sonication process, sedimentation was observed during the first 15 min of sitting undisturbed at room temperature. Shortly after sonication, sedimentation of MWCNTs was observed at the bottom of the small vials, and most of the MWCNTs were settled after 15 min of bath sonication in Milli-Q water (Figure 2.19 A), as well as after bath sonication in the OECD 201 medium (Figure 2.20 A).

However, after probe sonication in Milli-Q water (Figure 2.19 B), MWCNTs were seen to persist in the water column, which is a good indicator that the MWCNTs were well dispersed (Figure 2.19 A). In contrast, a slight sedimentation was observed after

15 min of sitting at room temperature following probe sonication to disperse MWCNTs in the OECD 201 medium. The tubes were found to be well dispersed in the OECD 201 medium, forming a homogeneous suspension (Figure 2.20 B).

However, a large proportion of MWCNTs were settled after 24 h of probe sonication (Figure 2.20 B). Therefore, this study demonstrated that probe sonication was, in general, a more effective way of dispersing the MWCNTs, despite the settlement of the NMs in both treatments after 24 h of being left undisturbed. It is important to note that the concentration used was of the stock suspension, and that the two methods used reflect different standard methods, which could lead to different outcomes if different initial concentrations and sonication times are used.

2.3.3.1 Assessment of the stability of MWCNTs

The settling behaviour of MWCNTs (100 mg/L) was studied immediately after the sonication process. The MWCNTs were bath sonicated or probe sonicated in Milli-Q water and in OECD 201 medium. A volume of 2.7 mL of this suspension was immediately transferred into a cuvette (1 cm path length) and absorbance measurements were taken at 660 nm (Zhang et al. 2014) at intervals of 5 min for 120 min, then at 24 h, using a spectrophotometer (Jenway model 6715). The results obtained showed a rapid reduction in both media (Milli-Q water and OECD 201 medium), measured by optical density during the first 15 min after bath and probe sonication. However, after using bath sonication in both media (Milli-Q water and OECD 201 medium), the absorbance of MWCNTs was steady at around 0.2 and 0.4 OD, respectively (Figure 2.21). A significant decrease in OD was observed during the first 15 and 30 min for bath sonication, in Milli-Q water and OECD 201 medium, respectively.

For the probe-sonicated samples, a decrease in OD measurements was observed during the first 50 min in Milli-Q water. Interestingly, a decrease in OD measurements was observed during the first 20 min for probe-sonicated samples in OECD 201 medium. It can be observed that after probe sonication in OECD 201 medium the suspensions appeared to stabilise around 1.1 OD, as evidenced from the plateau shape of the settling curve. Moreover, the settling curves showed that probe sonication in OECD 201 medium slightly increased the stability of the MWCNTs' dispersion (Figure 2.21). Generally, probe sonication for MWCNTs in OECD 201 medium increased the stability of the MWCNTs' dispersion > probe sonicated in Milli-Q water > bath

sonication in OECD 201 medium > bath sonication in Milli-Q water. Moreover, probe sonication increased the stability of MWCNTs in the dispersed solutions compared to bath sonication.

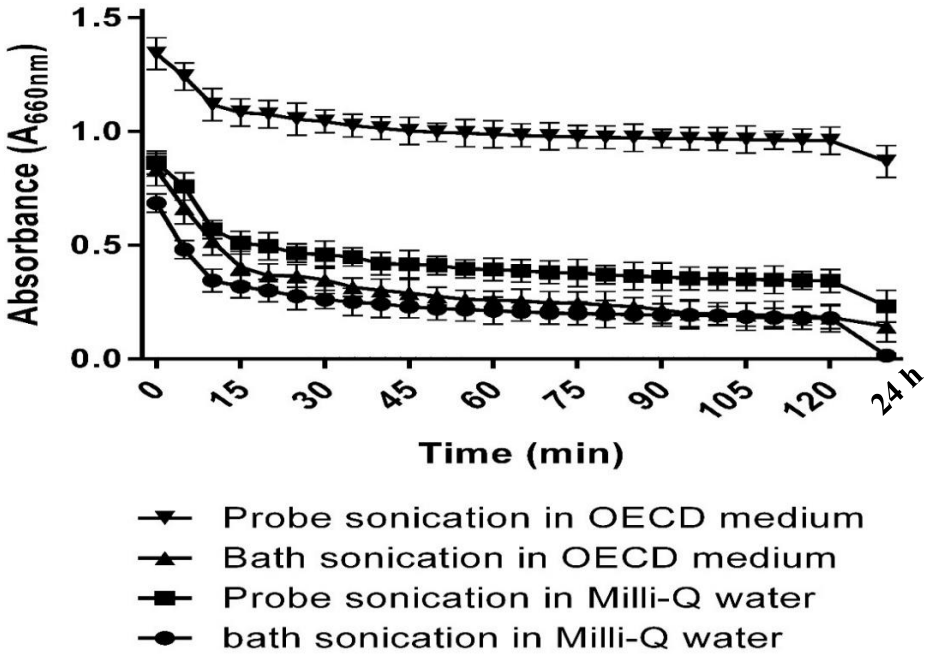


Figure 2.21: Stability of 100 mg/L MWCNT dispersions in Milli-Q water or in OECD 201 medium during 120 min immediately after the initial stage of sonication (bath or probe), then after 24 h as monitored via UV–Vis spectrophotometer at 660 nm. Optical density was measured every 5 min for 120 min, then at 24 h (n=3).

2.4 Discussion

A thorough physicochemical characterisation of NMs in parallel to hazard investigations can help identify which NM attributes are responsible for any observed toxicity (Johnston et al. 2013). The physical and chemical properties of Ag NMs, including surface charge, size, size distribution, shape, particle morphology, particle composition, coating/capping, and agglomeration are crucial factors for the determination of toxicity (Carlson et al. 2008; Warheit 2008; Zhang et al. 2011; Sriram et al. 2012; Gliga et al. 2014). This chapter (NM characterisation) used DLS in conjunction with TEM to evaluate the hydrodynamic diameter and zeta potential for Ag NMs as well as the size and state of the dispersion for Ag NMs and MWCNTs that had been dispersed in OECD 201 medium using two different types of sonication (bath and probe) prior to *in vivo* toxicology experiments. Furthermore, MWCNTs were not suitable for analyses by DLS, as only spherical particles are suitable for DLS measurements (Malvern Instruments Ltd 2013). Ag NMs and MWCNTs were additionally analysed by TEM, using two different methods.

The importance of NM characterisation is not limited to NM risk evaluation, but also to the importance of assessing the fate of NMs in media during assays, as well as to linking the properties of NMs to their potential toxic effects (López-Serrano et al. 2014). Characterisation has now become an essential aspect in the studies of NM toxicity. Therefore, the work in this chapter was performed to gather information of Ag NMs and MWCNT properties under test conditions, which is important in the assessment of NM risks.

2.4.1 *DLS*

2.4.1.1 Hydrodynamic diameter

DLS was used to obtain size and charge characterisation of Ag NMs in OECD 201 medium. Measurements were conducted with a Zetasizer Nano Series (Malvern Instruments, Malvern, UK) in a plastic measuring cuvette (DTS1070, Malvern Instruments) and at least three concordant measurements were recorded to calculate a mean z average size. DLS measurements were made on all tested concentrations at t=0, 24, 48 and 72 h.

Measurements of NMs' hydrodynamic diameters using DLS were shown to be dependent on the medium used for the sample preparation (Klein et al. 2011). While the characterisation of stock suspension can give information about the initial proprieties of NMs, some of these parameters change when the NMs are added to the exposure test medium (Kleiven et al. 2018). Therefore, a range of tested concentrations, based on the toxicity tests conducted in this project, were made and analysed by DLS to understand the behaviour of NMs in OECD 201 medium, using both types of sonication (bath and probe). The concentrations used in the Ag NMs study were 20, 50, 80, 150, 200, 250 and 300 µg Ag/L, which represent the actual concentrations used in the toxicity experiments (Chapter 3). The results showed that the mean zeta-average particle size in all the tested concentrations was close, with a slight increase over the experiment time points.

The findings showed that the Ag NMs hydrodynamic diameters for all tested concentrations and across time points were between 92.4 and 217.6 nm, and between 78.06 and 215.73 nm for bath and probe sonication, respectively. This aligns with the hydrodynamic diameters of Ag NMs particles observed in the published literature, in a variety of media, such as human cell culture (Kermanizadeh et al. 2013; Johnston et al. 2015), bacterial studies (Losasso et al. 2014; Mallevre et al. 2014; Donnellan et al.

2016), and environmental media (Wang et al. 2012; Völker et al. 2013; Kleiven et al. 2018). Furthermore, DLS was a useful technique to explain the behaviour of Ag NMs. It was observed that there was an agglomeration of Ag NMs in the OECD 201 medium over the experiment time, and this finding was also observed by Donnellan et al. (2016) using different media (Middlebrook 7H9), and confirmed by Kleiven et al (2018) using Milli-Q water to disperse Ag NMs. Moreover, the findings in this research study are in agreement with the findings of Wang et al. (2012) using a different test medium. Wang et al. (2012) found that the particle size of Ag NMs had a wide distribution in their test medium. The range of particle sizes observed by these authors was between 95 and 210 nm, which is significantly larger than the primary size provided by the manufacturers (Wang et al. 2012). Depending on the various protocols used, different results may be obtained for the same parameter. The effects of an NM's "corona", and the molecules surrounding it in a given medium, have also been acknowledged (Cedervall et al. 2007), emphasising that the constituents of the corona depend on the medium.

Generally, it can be concluded that the probe-sonication dispersion method was a better way to prepare highly dispersed NM suspensions, compared to bath sonication. Indeed, the difference in the NMs' agglomeration was a result of the efficiency of probe sonication to disperse the NMs rather than bath sonication.

2.4.1.2 Zeta Potential

The zeta potential measured in this study is similar to published literature; negative but >-25 mV (Wang et al. 2012; Völker et al. 2013; Sorensen and Baun 2014; Donnellan et al. 2016; Kleiven et al. 2018). Ag NMs had a negative zeta potential charge using both types of sonication (bath and probe) and the values were in the range of -4.76 to -24.6 mV over the experiment time. In an electrostatically stable suspension, particle zeta potential must be sufficiently high (outside the range from -25 to +25 mV) to overcome weak attractive forces (Hanaor et al. 2012; Malvern Instruments Ltd 2013).

Similar particle properties were found across a number of different characterisation studies (Klein et al. 2011; Kermanizadeh et al. 2013; Völker et al. 2013; Mallevre et al. 2014; Sorensen and Baun 2014; Johnston et al. 2015; Donnellan et al. 2016), which indicate that Ag NMs tend to be relatively well dispersed in a range of media. Moreover, the findings of this research study are in agreement with the findings of Wang et al. (2012) using zebra fish medium and Sorensen and Baun (2014) using ISO

algal medium. Wang et al. (2012) found that the zeta potential of Ag NMs was -10.3 to -13.9 mV. Sorensen and Baun (2014) indicated that Ag NMs had a negative charge, with a zeta potential value of -21 ± 4.3 mV. Furthermore, Völker et al. (2013) indicated that the zeta potential of Ag NMs was -17.0 ± 0.57 mV in OECD 202 M4 medium.

The zeta potential of NMs is related to the pH of the suspension, with higher pH (more alkaline) generally causing a larger negative charge, and with lower pH (more acidic) a more positive one (Ghosh et al. 2008; Peretyazhko et al. 2014). As described in Chapter 3, the OECD 201 medium has a pH of ~7.8, so a slight increase in the negative charge in the zeta potential was observed in the OECD 201 medium. Overall, based on the obtained results from zeta potential experiments, the probe-sonication method has a greater effect on the zeta potential of NMs than the bath-sonication method.

2.4.2 *TEM*

2.4.2.1 Ag NMs

To visualise and support the determination of the size/dimensions of Ag NMs, as well as to examine the presence of NM agglomerates, samples of Ag NMs suspended in OECD 201 medium were analysed to determine their natural state in the relevant medium without including other additional factors such as salts or organic matter. For the TEM sample preparation, two different approaches were used: drop casting and plunge freezing of the NM stock suspension (10 mg/L). Ag NMs morphology had a spherical structure after bath and probe sonication, and there was no observable effect of the sonication method on the structure or morphology of Ag NMs. These findings are in accordance with other published studies which investigated the morphology of Ag NMs in different media using TEM (Klein et al. 2011; Wang et al. 2012; Kermanizadeh et al. 2013; Losasso et al. 2014; Sorensen and Baun 2014; Donnellan et al. 2016; Kleiven et al. 2018). The general shape of the Ag NMs was spherical, with the sporadic presence of regular polygonal NMs, which was also reported previously by Kermanizadeh et al. (2013) and Völker et al. (2013).

It was observed, by using drop casting for TEM sample preparation, that spherical Ag NMs tended to attach to each other as agglomerates in the OECD 201 medium, whereas the Ag NMs were observed as more dispersed when using the plunge freezing procedure. Moreover, in the case of probe sonication, a better dispersion was observed compared to bath sonication.

The bath- and probe-sonicated Ag NMs were confirmed to have a diameter of approximately 20 nm, as stated by the supplier (Klein et al. 2011). The TEM analysis for the Ag NMs showed a good agreement with the manufacturer's specifications (Klein et al. 2011), whereas the DLS measurements indicated a higher mean particle size when tested in OECD 201 medium. Ag NM toxicity mainly depends on the availability of chemical and/or biological coatings on the NM surface (Suresh et al. 2012). Moreover, Ag NM surface charges could determine their toxicity effect in cells.

2.4.2.2 MWCNTs

It was found that the bath-sonicated MWCNTs formed agglomerates in OECD 201 medium when using both methods for TEM sample preparation. In contrast, in the case of probe sonication, it was noticed that there was a lower agglomeration level, especially when using the plunge-freezing process for TEM sample preparation. Furthermore, MWCNTs appeared to contain a fraction of micrometre-sized particles or crystals. Generally, MWCNTs appeared to consist of entangled, irregular, and bent nanotubes, and it was difficult to measure the dimensions of these nanotubes.

One of TEM's disadvantages is limited applicability when investigating hydrodynamic processes due to the fact that samples must be dried, which can in turn introduce artefacts such as misidentification of salts, or false agglomeration during evaporation (Domingos et al. 2009; Pettitt and Lead 2013). However, there are different procedures for TEM sample preparation, such as plunge freezing, which gives good representative images for the agglomeration stats (Hondow et al. 2012).

The findings from this chapter confirmed that the plunge freezing process is a good procedure for TEM sample preparation compared to the drop casting procedure. This finding was in agreement with Hondow et al. (2012) who reported that plunge freezing for NM dispersions is a viable option for TEM imaging and quantification of NMs in their dispersed state. Furthermore, based on previous observation, this indicates that probe sonication is more efficient in dispersing the agglomerates of MWCNTs.

Some toxicological studies support the assertion that smaller-sized particles could be more toxic than larger NMs, because they have a larger surface area (He et al. 2010; Sriram et al. 2012; BeigzadehMilani 2015; Sabry and Ragaei 2018; Kanwal et al. 2019; Saleeb et al. 2019). Shape is equally important to the determination of toxicity (Stoehr et al. 2011). Moreover, EDX analysis is helpful to distinguish between

different NMs based on their elemental components. However, the quantification of a low concentration of dissolved atoms is not straightforward (Markwitz et al. 1998).

In terms of MWCNTs, previous authors (Huang et al. 2002) have stated that depending on the duration and type (bath or probe) of sonication, nanotubes might be shortened to different lengths in the functionalisation reactions. A shortened MWCNT length reduces the aspect ratio and deteriorates the mechanical and electrical properties of MWCNTs (Park et al. 2012). Probe sonication allows for a relatively efficient preparation of suspended CNTs of a potentially selectable average length (Li et al. 2007; Huang et al. 2002; Meija et al. 2011). Huang et al. (2002) used probe sonication to change the surface properties of MWCNTs. In Huang et al.'s study, CNTs were prepared via functionalisation with poly(propionylethylenimine-co-ethylenimine) (PPEI-EI). The diimide-activated amidation reaction for the functionalisation was found to be significantly improved in both efficiency and yield by sonication under ambient conditions. Moreover, it was also found that depending on the duration of sonication, the nanotubes were shortened to different lengths in the functionalisation reactions (Huang et al. 2002). The probe-sonication method allows for a relatively efficient preparation of CNTs of a potentially selectable average length and shortened to different lengths of MWCNTs with changed surface properties; all these factors may lead to an increase in the degree of toxicity, which is due to artefacts arising from the dispersion method, which is not helpful to disperse CNTs.

Different approaches for the assessment of NM characterisation, as well as differences between sampling protocols, concentrations and time points tested, combined with the possibility of drying artefacts (Tauer 2004; Domingos et al. 2009), could account for differences in the agglomeration status of NMs observed in different studies. Results from MWCNTs characterisation studies differ in regards to diameter, agglomeration size and impurity content (Kermanizadeh et al. 2013; Vietti et al. 2013; Cao et al. 2014).

2.4.2.3 EDX

The role of EDX was to identify the Ag metal in TEM samples, as well as the purity of NMs in general (Brown et al. 2014; Gliga et al. 2014; Klingberg et al. 2015). Surface morphology and the presence of impurities in Ag NMs and MWCNTs were determined by TEM and EDX. The MWCNTs had impurities on their surface and thus EDX could be used well to track their fate in aquatic medium. Elemental analysis using EDX indicated that MWCNTs contained residual Fe, Al, Cu, Co, S, Na and Si

and corundum crystal content originating from the catalyst used during the synthesis of MWCNTs. Rasmussen et al. (2014) reported a similar finding of metal residues on MWCNTs, and attributed these residues to poor dispersion of the metal catalyst. Moreover, Hougaard et al. (2013) and Öner et al. (2016) reported some different amounts of element impurites associated with MWCNT (NM400) such as Al, Fe and Co. The presence of Fe impurities has been linked to toxicity due to its role in the formation of free radicals and ROS (Shvedova et al. 2003; Nel et al. 2006), and the activity of ROS tends to be proportional to the concentration of Fe impurities in CNT suspension (Kagan et al. 2006). Metal catalyst residues are often very difficult to remove from carbon-based NMs (Lukhele et al. 2015). These metals are often embedded in the tube and their removal involves using harsh acid treatment which can destroy the nanotube (Lukhele et al. 2015).

2.4.3 *Visual examination*

The settling behaviour of MWCNTs (100 mg/L) was studied immediately after the sonication process. The MWCNTs were bath sonicated or probe sonicated in Milli-Q water and in OECD 201 medium. Generally, the stability of MWCNTs was found to decrease in the following order: probe sonicated in OECD 201 medium > probe sonicated in Milli-Q water > bath sonicated in OECD 201 medium > bath sonicated in Milli-Q water. In general, the results show that the sonication type seems to have an important influence on the degree of dispersion and final NM agglomeration in OECD 201 medium.

2.5 Conclusion

The differences in characterisation data are likely driven by factors such as test medium, time point, samples preparation method, type of sonication, NM concentration and the characterisation method. For studies which cross validated DLS data using TEM, it was found that both methods showed similar estimates of size for Ag NMs (Kermanizadeh et al. 2013; Losasso et al. 2014; Johnston et al. 2015; Donnellan et al. 2016; Kleiven et al. 2018). For Ag NMs and MWCNTs, probe sonication clearly disperses NMs into smaller and more monodisperse units, as judged by DLS measurements (for Ag NMs) and TEM images (for Ag NMs and MWCNTs) compared with the bath-sonication procedure. Overall, probe sonication resulted in the most homogenously sized material dispersions. These findings are in line with previous observations (Nickel et al. 2014; Pradhan et al. 2016).

Dispersion stability is the ability of a dispersion to resist changes in properties over time, and it is an essential factor in the toxicity testing of NMs (Rasmussen et al. 2018). Al-Shaeri et al. (2013) suggested that CNTs simply precipitate and accumulate in sediments. The status of dispersion influences the results of any test that requires the sample to be in suspension, as the NMs likely agglomerate in the test medium (Rasmussen et al. 2018). This situation might occur because the repulsive forces are not strong enough to keep the NMs sufficiently separated to prevent short-range attractive forces becoming dominant (Rasmussen et al. 2018). The NMs might also be stabilised by stabilisers present in the media or NM surface. The determination of dispersion stability is fundamental prior to any toxicity testing, especially if the NMs have to be dispersed in the test medium before administration (Rasmussen et al. 2018). Changes in the dispersion stability affect physical properties that, in turn, can affect the hazard properties and environmental fate of NMs (USEPA 2017).

Moreover, the findings show that the sonicator seems to have an important influence on the degree of dispersion and final particle agglomeration in the OECD 201 medium. For studies which cross validated DLS data using TEM, it was found that both methods showed similar estimates of size for Ag NMs (Kermanizadeh et al. 2013; Losasso et al. 2014; Johnston et al. 2015; Donnellan et al. 2016; Kleiven et al. 2018). Overall, probe sonication resulted in the most homogenously sized particle dispersions. These findings are in line with previous observations (Nickel et al. 2014; Pradhan et al. 2016). The advantage of the use of probe sonication instead of bath sonication is that the probe sonicator delivers the energy directly to the samples, while in bath sonication, the energy is partly absorbed by the liquid (for example water) in the bath, and this depends on the volume of liquid. Moreover, the material container in which the sample is placed, such as glass or polyethene, can have an impact on energy delivery to the samples. When NMs are dispersed in environmental media, their fate may be different from, and potentially more complex than, what may be found in biological media (Hondow et al. 2015). Moreover, it would be unrealistic and impractical to offer a unique set of sonication conditions for all possible systems; the ultimate goal of the optimisation procedure should be to achieve the desired degree of particle dispersion with the least possible energy input, in order to minimise unwanted side effects (Taurozzi et al. 2011).

Chapter 3

Acute toxicity of NMs to algae

Chapter 3 **Acute toxicity of NMs to algae**

3.1 Introduction

Standard tests are available to assess the aquatic toxicity of chemicals, however, there is concern that they may not be suitable for assessing the toxicity of NMs. OECD has set out guidelines to standardise methods used to assess the aquatic toxicity of chemicals. Test guidelines are available for a range of model organisms (including crustaceans, fish, algae etc.). Algae are used to assess the impact of chemicals on the aquatic environment; for example, *R. subcapitata* and *C. vulgaris* (Schwab et al. 2011), *Chlorella sp.* (Long et al. 2012), *Chlorella pyrenoidosa* (Zhang et al. 2014), *R. subcapitata* (Lukhele et al. 2015), *R. subcapitata* and *C. vulgaris* (Sohn et al. 2015), *Desmodesmus subspicatus* (Rhiem et al. 2015) and *R. subcapitata* (Brown et al. 2018). Currently, the methods used for analysing and quantifying extracted biomass pigments, including automated counting, traditional microscopy and measuring fluorescence, are not seen as particularly accurate in the assessment of the effects of NMs since different types of NMs have dissimilar environmental behaviours and are themselves diverse. Furthermore, the composition of the medium used can influence NM behaviour. Biomass concentration, the presence of organic matter, and ionic strength can affect the aggregation and/or agglomeration of NMs (Keller et al. 2010). Keller et al. (2010) indicated that TiO_2 NMs in an aqueous medium begin to agglomerate immediately, as do gold NMs (Au NMs) when coated with stable colloidal dispersions (Daniel and Astruc 2004).

One important parameter in ascertaining and measuring the performance and productivity of microalgae is their biomass concentration, which is affected by the properties of the cell, either biological, physical or chemical.

When considering industrial chemicals and their hazard classifications, algae growth inhibition is more responsive than either fish or crustaceans, therefore it is a more accurate test species to use (Weyers and Vollmer 2000). Therefore it is often suggested that algae are used, to assess the aquatic toxicity of chemicals (Blaise et al. 1986).

3.1.1 *Quantification of algal biomass*

There are several techniques which can be used when measuring the cell density of microalgae, including: dry cell weight; manual cell count; automatic cell count; spectrophotometry and optical density (OD); near-infrared optical density; oxygen

production rate (OPR); dielectric permittivity, and the Red-Green-Blue (RGB)-model imaging analysis in combination with Fourier's equation and flow cytometry (FCM) (Strickland and Parsons 1972; Hallegraeff 1977; Sournia 1978; Stein 1979; Coutteau 1996).

When conducting inhibition tests on algal growth, the most common response endpoints used are the growth rate and the final yield biomass (cell density) at the end of the exposure period (OECD, 2011).

Analysis of final yield biomass results from the International Organization for Standardization's (ISO) ring tests showed that experimental results standing on final yield tests overall were greater than those standing on growth rates (Hanstveit 1982). From a theoretical point of view, however, as growth rate is less dependent on any particular test's system parameters, it is seen as a more accurate response variable than final yield cell density (Nyholm 1985).

Providing fast, simple and accurate measurements of an algal's biomass concentration is crucial when setting out to analyse and improve the microalgae culture system's performance (Sandnes et al. 2006) as well as for assessing the toxicity of chemicals. If a large range of cell concentrations is used, the measured response should be linear over time, with a measurement system in position which is able to perform online analysis (Havlik et al. 2013).

Cell concentrations can be determined in two ways: either by measuring a cell's mass, or by counting the number of cells. The former is best used when there is an abundance of samples to analyse, as direct biomass weighing, where the cells are dissociated from the broth and dry weighed, is a reliable and straightforward process. An example of the latter is FCM, which has the advantage of allowing simultaneous analysis of several outcome variables and their chemical and physical characteristics (Hyka et al. 2013). Its main disadvantage, however, is the requirement for expensive equipment and expertise (Marie et al. 2005).

The most common approach to monitor algal growth is assessment of optical density, which uses light absorption to ascertain how much light, and which wavelengths, pass through a given material on to a detector (Thatipamala and Hill 1991). This method becomes inconsistent, however, if there is some insoluble material (such as NMs) in the culture, or if coloured/opaque holophytic algae are used (Kell et al. 1990).

Tibayrenc et al. (2011) investigated the effect of technological stress on *Saccharomyces cerevisiae* during alcoholic fermentation by using dielectric

permittivity as a technique for biomass measurement. This technique showed that not all cells in the culture are counted in the dielectric permittivity measure; non-polarisable cells, such as plasma membrane, cell debris, and dead cells which have a permeabilised plasma membrane are not counted (Tibayrenc et al. 2011). As this technology (dielectric permittivity) is still relatively new, its reliability has not been scientifically evaluated.

An alternative to using optical density for estimating algal biomass is via quantification of chlorophyll concentration using a spectrophotometer (Su et al. 2008). Since microalgae's photosynthetic pigments absorb only specific wavelengths of visible light, while reflecting others, depending on their composition, these pigments can be used to estimate biomass, using a classifier algorithm (Coltelli et al. 2013).

Another method recently championed is using image analysis (Sarrafzadeh et al. 2015). At this point, RGB lights are passed through a microalgal biomass, and images are taken from the sample. As different light refracts at different wavelengths, biomass concentrations can be calculated through the correlation between biomass concentrations and different light refractions. This method is relatively quick and inexpensive.

There are other methods which can also give algal cell biomass estimates: the separate components, such as DNA, ATP, proteins and lipids can be examined and the biomass content estimated. However, as these methods require the use of chemicals and solvent extraction, they can be expensive and laborious (Hurst et al. 2007; Ördög et al. 2012). Thus, the different characteristics of each method have some advantages and disadvantages, and this study uses the estimation of algae biomass/activity using the following methods:

(i) Chlorophyll extraction (Chl),
(ii) Optical density (OD),
(iii) Protein content and
(iv) Photosystem II activity (PSII).

3.1.2 *Chlorophyll extraction (Chl)*

Chlorophyll (Chl) is a photosynthetic pigment found in the chloroplast of most plant and alga cells, and is present in all green plant life (Hosikian et al. 2010; Siddiqui et al. 2015). Chl uses light from the visible spectrum, primarily absorbing blue, violet and red light while reflecting green light, which is what gives it its green appearance.

By measuring Chl, one can find the quantity of algal biomass active in a specific area, such as a stream bed, and one can also determine how much phytoplankton is present in a sample of water.

During a cell's life cycle, different amounts of Chl are produced (Hosikian et al. 2010). Hence, since the carbon content remains the same, the carbon-to-Chl ratio will change significantly depending on how healthy the algal population is. As a result, this ratio can be used as a measurement for algal biomass. When dry weight was utilised for algal biomass determination, it was found there was too much interference from particles, both with *in vivo* measurement of Chl concentrations by fluorescence and when counting the cells (Handy et al. 2012a; Handy et al. 2012b; Hartmann et al. 2013; Hund-Rinke et al. 2016; Farkas and Booth 2017).

The problem of the fluorescence signal effect is significant as a source of interference. Hartmann et al. (2013) and Hund-Rinke et al. (2016) suggested separating the Chl from the target algal cells, and extracting the NMs before an *in vitro* fluorescence measurement of the algal extract. Other methods proposed include: using acetone extraction (Mayer et al. 1997; Sørensen et al. 2016); ethanol extraction and filtration (ISO 1992), and employing locust gum, extracting acetone with NM flocculation and sedimentation (no filtration) (Kalman et al. 2015; Cerrillo et al. 2016; Hund-Rinke et al. 2016). Furthermore, the extracting of Chl can be done in acetone (80%) and then collecting the supernatant and measuring absorbance at 652 nm with a UV–Vis spectrophotometer (Dash et al. 2012).

3.1.3 *Optical density*

A measurement of optical density is often used to ascertain the level of biomass for unicellular organisms, as this method is cheap, quick and non-destructive (Toennies and Gallant 1949; Shuler and Kargi 2005).

The level of light absorbed is directly related to cell mass and/or cell number in a suspension of cells. The absorbance of particular wavelengths and cell concentrations can be plotted on a standard curve using the wavelength of light that has maximum absorbance, which gives the widest range of sensitivity (Griffiths et al. 2011). Mistakes in the assessment of biomass concentration can occur if any of cell morphology, optical properties or composition are altered.

Algal culture media are normally transparent, and in the literature, there is some agreement about which wavelengths of light can be utilised for microalgal cultures

(Griffiths et al. 2011). The largest signal can be found by using a wavelength that lies between the pigment's maximal absorbance range, with experiments showing this is normally within the ranges of 400 to 460 nm and 650 to 685 nm (Linschitz and Sarkanen 1958; Piorreck et al. 1984; Sung et al. 1999; Takagi et al. 2000; An et al. 2003; Bopp and Lettieri 2007; De Morais and Costa 2007; Chiu et al. 2008; Hsieh and Wu 2009).

In one study conducted by Rodrigues et al. (2011), the spectrophotometry was used to evaluate the algal density. The unicellular algae *R. subcapitata* was used in this study. A calibration curve was plotted based on the changes in algal density by scanning a culture sample between 600 and 800 nm. The algal culture was inspected between the lowest absorbance of algal density from 1 million cells/mL up to the maximum absorbance at 5 million cells/mL, with a high coefficient (r2 = 0.9998) (Rodrigues et al. 2011).

Another study was carried out by Rodea-Palomares et al. (2011), who investigated the ecotoxicological effects of CeO_2 NMs on *R. subcapitata*. The growth inhibition of *R. subcapitata* was assessed by measuring the OD of algae cultured in a microplate, in parallel with cell counting. The observed effect on the growth rate of *R. subcapitata* when using OD was particularly high. Furthermore, the IC_{50} values were low because of the formation of agglomerates of algal cells with CeO_2 NMs. In another study on the effect of Ag NMs on *R. subcapitata*, Książyk et al. (2015) described the inhibition of algal growth after 72 h of exposure relative to controls by measuring optical density at 670 nm. The commercial Algaltoxkit FTM (Creasel, Belgium) was used and the initial density of cells was 10^6/mL. Their results confirmed that a concentration $\geq$5 mg/L of Ag NM caused a 100% drop in algal biomass.

3.1.4 *Protein content*

Green microalgae usually contain approximately 25–50% (w/w) of the algal biomass as protein (Belitz et al. 2009; Harnedy and Fitzgerald 2011; Schwenzfeier 2013). Determination of protein content of algae can be used to assess algal biomass.

There are several methods for protein extraction which can be applied to algae; these methods include enzymatic hydrolysis to prevent the degradation of proteins (Fleurence 1999; Joubert and Fleurence 2008), physical processes such as cell disintegration, e.g. by sonication or homogenisation (Barbarino and Lourenço 2005),

and chemical extraction (Jordan and Vilter 1991; Fleurence et al. 1995; Kadam et al. 2017).

One of the most commonly used methods for quantifying protein in microalgae is the blue dye method as it called Bradford assay (Bradford 1976). This method is based on bovine serum albumin (BSA) as a standard protein. The BSA is a serum albumin protein derived from cows, usually used as a protein concentration standard in lab experiments. In the Bradford assay, the dye binds to basic and aromatic amino acid residues (Kaehler and Kennish 1996). The Lowry method (Lowry et al. 1951) uses a colourimetric approach which measures protein (Lourenço et al. 2004). Moreover, there are some novel protein-extraction methods, including ultrasound-assisted extraction, pulsed electric field, and microwave-assisted extraction (Kadam et al. 2013). Recently, a procedure for algal protein fluorometric measurement has been developed, which has the advantage of only using very small samples, and has increased throughput potential (Kadam et al. 2013). The procedures of calorimetry and fluorimetry are vulnerable to interferences; both from extraction buffer constituents and non-protein cellular components, and are also conditional on the efficiency of the solubilisation of the cellular proteins (cell fractionation). These procedures are also heavily dependent on the protein standard used for calibrating the absorbance/ fluorescence values (Laurens et al. 2012).

Classical procedures for extracting the algal proteins are inhibited by the cell wall's mucilage (Fleurence et al. 1995). These polysaccharides, both neutral and anionic, have a limiting effect on the efficacy of extracting the amino acids (Fleurence et al. 1995). Some unicellular microalgae, including *Tetraselmis sp.*, have strong cellulosic cell walls, which inhibit intracellular proteins from being extracted (Becker 2007; Doucha and Lívanský 2008; Schwenzfeier 2013). In order to extract the intracellular proteins, these cell walls must be degenerated using a harsh organic chemical, such as agarase or by incorporating enzymes that have cell-wall-degrading properties. As Fleurence (1999) observed, however, as every species of algae has a dissimilar cell wall composition, each sample would require independent validation. Additionally, only enzyme preparations which have no proteolytic side activity should be considered, to avert protein degradation. Schwenzfeier et al. (2011) suggested using homogenisation or sonication to encourage cell degradation, although a further precipitation step was required to encourage protein purity.

Jiang et al. (2014) investigated the effect of 0.5–10 mg/L of Ag NMs on the protein content of higher plants (*Spirodela polyrhiza*) by the Bradford method. Their findings confirmed that the protein content increased when the concentration reached a maximum Ag concentration of 5 mg/L, and then decreased at 10 mg/L. Moreover, their findings confirmed that the highest concentration of Ag NMs decreased the protein content, indicating that a high concentration of Ag NMs damaged protein generation or accelerated protein decomposition because of oxidation (Jiang et al. 2014). Furthermore, they suggested that Ag NMs may penetrate into the tissues of *S. polyrhiza* and bind the proteins, therefore affecting physiological functions, including inducing oxidative stress (Jiang et al. 2014).

3.1.5 *Photosynthetic activity (PSII)*

Photosynthesis is a chemical process that uses solar energy to convert carbon dioxide (CO_2) into different organic compounds (Najafpour and Pashaei 2012; Domingo et al. 2019). Photosynthesis happens when light falls on the chloroplasts of any green plant or algae, creating energy inside the cell, through producing reactive oxygen species (ROS) (Asada 2006; Jaspers and Kangasjärvi 2010).

The photosynthetic rate can be determined by using different methods, such as: an infrared gas analyser (Chen et al. 2012c); analysing the concentration of photosynthetic pigments in algal cells (Książyk et al. 2015); fluorometry using a PHYTO-PAM (Heinz Walz GmbH) equipped with an Optical Unit ED-101US/MP (Navarro et al. 2008b).

As Jiang et al. (2014) showed, a change in chloroplast ultrastructure caused by ROS production is induced by Ag NMs in *S. polyrhiza*. This is particularly true of the aquatic plants such as *Spirodela polyrhiza*, where Ag NMs have a direct effect on chloroplasts. There have been many studies into the effects of Ag NMs on algae (such as *D. tertiolecta* and *C. vulgaris*) which have shown Ag NMs having an adverse effect on photosynthesis, and a resultant decrease on PSII efficiency (Navarro et al. 2008b; Oukarroum et al. 2012b).

Navarro et al. (2015) investigated the effects of different coated Ag NMs and $AgNO_3$ on the photosynthesis of the freshwater algae *Chlamydomonas reinhardtii*. The authors were able to confirm that in all cases, the excess cysteine completely prevented the effects of Ag NMs on photosynthetic yield compared to $AgNO_3$. This finding confirms the role of Ag ions as a cause of the observed effect on the photosynthesis.

Moreover, the PSII of *C. reinhardtii* is affected, to a greater degree and when under an Ag NM treatment, when in light as opposed to dark conditions (Dewez and Oukarroum 2012b).

In general, the typical method for assessing algal biomass has to provide an accurate, rapid cell count, a low limit of interference with different materials in the sample, and must use a small number of samples. Graphically, the response, if over a sufficient range, will be linear, although different biomass samples will produce a different pattern. Moreover, dispersability and uniformity of dispersion are very important criteria that must be met in order to successfully determine the toxicity of NMs. It will therefore be essential to compare different dispersion methods to generate a relevant protocol that can be used to standardise assays and aid their interpretation (Stone and Donaldson 2006). Therefore, the effects of the two NM dispersion methods (probe and bath) are assessed on the acute effects observed using *R. subcapitata*.

3.1.6 *Aim and objective*

The main aim of this chapter is to investigate the acute toxicity of Ag NMs and MWCNTs to *R. subcapitata* using different methods, namely Chl a extraction, OD, protein content and PSII activity, when assessing the toxicity of NMs. The impact of the sonication procedure used to prepare NM dispersions on the toxicity to algae was evaluated in this study. Moreover, the interference of NMs with algal assays was assessed in OECD 201 medium in the absence of algal cells.

3.1.7 *Research hypotheses*

- Ag NMs will exhibit lower toxicity to *R. subcapitata* than ionic silver based on a total Ag concentration.
- Probe sonication for Ag NM suspensions will exhibit higher toxicity to *R. subcapitata* compared to bath sonication.
- Probe-sonicated MWCNTs will exhibit higher toxicity to *R. subcapitata* compared to bath-sonicated MWCNTs.
- NM toxicity results will not be different when using different methods for assessing algal biomass/activity.
- NMs will not interfere with algal effect assessment methods.

3.1.8 *Null hypotheses*

- There will be no difference in toxicity to *R. subcapitata* between NMs and ionic silver based on a total Ag concentration.
- There will be no difference in Ag NM toxicity to *R. subcapitata* between bath and probe sonication.
- There will be no difference in MWCNT toxicity to *R. subcapitata* between bath and probe sonication.
- There will be no differences in NM toxicity results to *R. subcapitata* using different algal biomass/activity methods.
- NMs will not interfere with algal effect assessment methods.

3.2 Methodology

3.2.1 *Preparation of glassware*

All glass test containers was prepared as previously described in Chapter 2, section 2.2.1.

3.2.2 *OECD 201 medium*

The OECD 201 medium was prepared according to the method outlined in methods for preparing OECD medium (see Chapter 2, section 2.2.2) (OECD 2011). Also, the constituents of the medium can be seen in Table 2.3.

3.2.3 *pH measurement*

It is known that toxicity of chemicals to *R. subcapitata* in standard OECD test medium 201 can be pH dependent (OECD 2011). The pH of the samples should not increase by more than 1.5 units in the course of the experiment period (0–72 h), as indicated in the OECD 201 guidelines (OECD, 2011). During all the experiments, the starting pH was 7.4 (± 0.8).

3.2.4 *Algal culture preparation*

The green alga species *R. subcapitata* (Strain 278/4) was acquired from the Culture Collection of Algae and Protozoa (CCAP), Oban, Scotland. The glassware used for the stock culture was autoclaved at 121 °C for 15 min prior to use. A volume of 1 mL of algal culture (25–30 × 10^4 cell/mL) was taken from the stock culture and mixed with 100 mL of OECD 201 medium. The algal density in the medium was measured using a spectrophotometer at a wavelength of 685 nm (A_{685nm}). The OECD 201

guideline state that the initial cell concentration of *R. subcapitata* for the starting culture should be 5×10^4 cells/mL (OD ~ 0.06) using a dual beam UV–visible spectrophotometer (Jenway model 6715) in a 10 cm cuvette at A_{685nm}. The reason for this biomass is to ensure there is a good algal density in order to allow the culture in the controls to grow exponentially during the test period.

3.2.5 Preparation of NM stock suspensions for ecotoxicological toxicity tests

The NMs (Ag NMs and NM300k DIS stock suspensions (Klein et al. 2011) and MWCNTs (NM400) powder (Rasmussen et al. 2014)) were obtained from the European Commission Joint Research Centre (JRC). The addition of a non-ionic polymer (e.g. polyoxyethylene (20) sorbitan mono-laurat and polyoxyethylene glycerol trioleate, in the case of Ag NMs DIS) can improve the stability of Ag NM suspensions. For more details about stock suspension preparation, see Chapter 2, section 2.2.4.

3.2.6 Sonication

Ag NMs and MWCNTs were bath and probe sonicated as previously described in Chapter 2, section 2.2.5.

3.2.7 *Exposure of algae to NMs*

Algae were prepared as described in section 3.2.4. Algae were exposed to OECD 201 medium (control, n=6) or OECD medium with NMs at 5 concentrations (n=3). The concentrations used for Ag NMs were 20, 50, 80, 150 and 200 µg/L for Ag NMs (following pilot studies) and 50, 100, 150, 200 and 250 µg/L for the Ag NMs dispersant (DIS). The concentrations for MWCNTs were 1, 5, 25, 45, 70 and 100 mg/L (following pilot studies). For the $AgNO_3$ studies, a stock solution of 100 mg/L was prepared using bath and probe sonication. The concentrations required were 3, 5, 10, 15, 20 and 25 µg/L (following pilot studies). Algae were exposed to the test substances for 72 h.

3.2.8 *Algal growth inhibition test*

Experiments were carried out in accordance with the OECD test guideline 201 for freshwater algae and cyanobacteria growth-inhibition tests (OECD 2011). For all experiments, 6 replicate controls and 3 replicates of each toxicant concentration were included.

Toxicant test concentration volumes were made in a 160 mL glass flask which included: 40 mL of the test substance ($AgNO_3$, Ag NMs, MWCNTs) at appropriate concentration (prepared in Milli-Q water), 40 mL of double-strength medium (OECD medium), and 80 mL of exponentially growing *R. subcapitata* diluted to 1×10^5 cells/mL. For control samples, 40 mL of Milli-Q water was added into OECD 201 medium and no toxicants were added. The test suspensions were then aliquoted into 3 of 250 mL borosilicate Erlenmeyer flasks; each flask contained ~ 53 mL sample (control or exposed sample) and was placed in an orbital shaker (Multitron Standard, Infors-HT, Multitron Pro) at 225 RPM, under continuous fluorescent light ~ 120 µmol/m^2/s, at 23 ˚C ± 2. Figure 3.1 summarises all stages of sample preparation, incubation and Chl a extraction and/or test measurements.

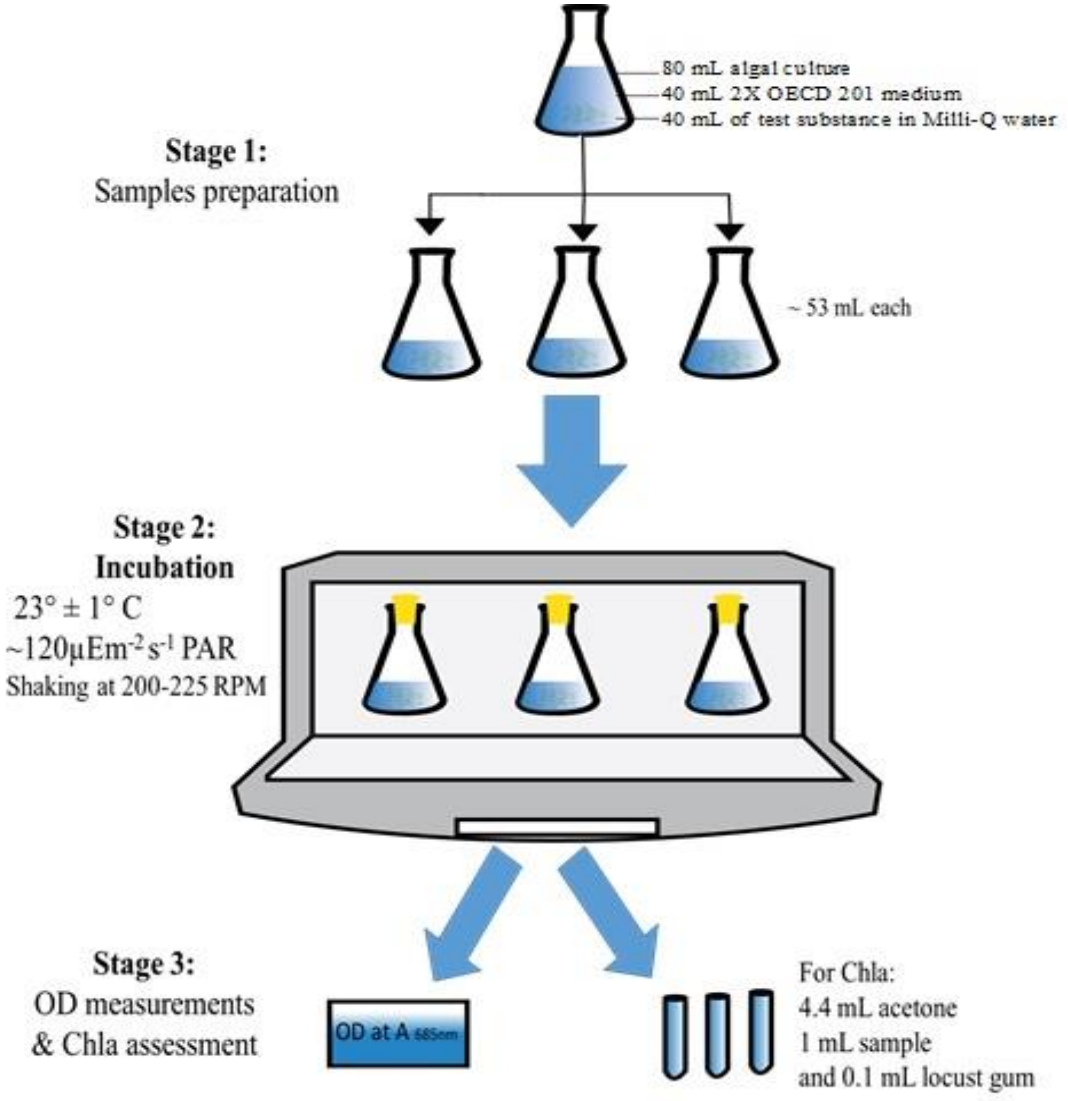


Figure 3.1: Stages of sample preparation, incubation and Chl a extraction and/or test measurements.

3.2.9 *Chlorophyll a extraction (Chl a)*

Complete chlorophyll extraction was performed in full-strength acetone (100%) in the dark to prevent photosynthesis activity, based on methods adapted from Mayer et al. (1997). A volume of 1 mL from each test substance algal suspension was added to a 100 µL locust gum solution (1.5 g/mL in Milli-Q water) in a 10 mL screw-cap glass tube. Locust gum was added to aid the settlement of cellular debris and particulates. Then 4.4 mL of full-strength acetone (100%) was added to the suspension to give a final acetone concentration in the samples of 80%. Samples were then left in the dark at 20° C. After 48 h, the supernatant was removed and then transferred to a cuvette and the concentration of chlorophyll was measured using a calibrated fluorimeter (Turner Design Instrument) using the Chl-NA module, and the concentration of Chl a was measured in µg/L. Solid state standards were used to check instrument drift periodically. Figure 3.2 displays the process flow diagram, showing the downstream processing steps required for chlorophyll extraction from microalgae.

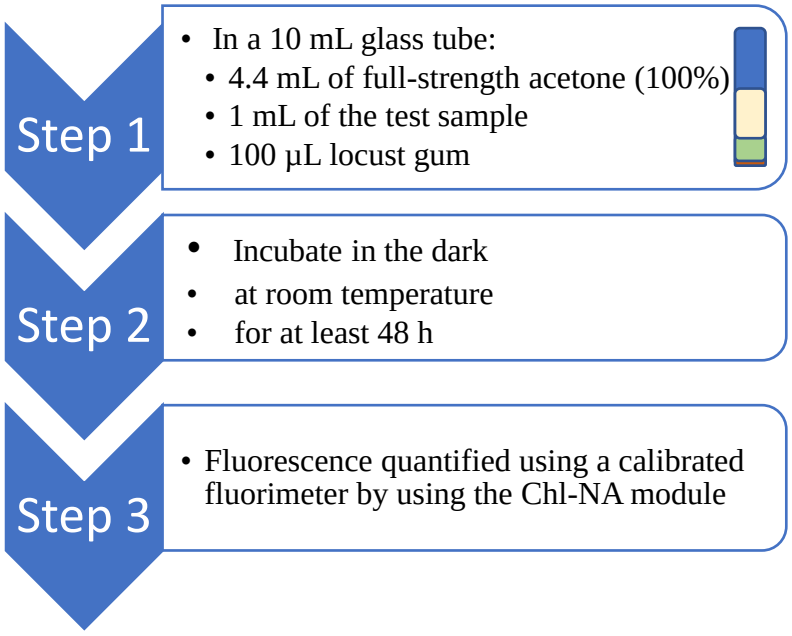


Figure 3.2: Process flow diagram showing the downstream processing steps required for chlorophyll a extraction from microalgae.

Raw data were treated following OECD 201 guidelines. In order to assess any growth inhibition, the growth rate for each concentration of each test substance was measured then compared with the control for each time point.

The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments:

$$\mu i - j = \frac{In\ Xj - In\ Xi}{tj - ti}\ (day^{-1}) \qquad (2)$$

where: $\mu i - j$ is the average specific growth rate from time i to j; Xi is the biomass (Chl a) at time i, and Xj is the biomass (Chl a) at time j.

The percentage inhibition of growth for each treatment replicate was calculated using the following equation:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \qquad (3)$$

where: $\%I_r$ is the percent inhibition, μ_C is the mean value for average specific growth rate in the control group and μ_T is the average specific growth rate for the treatment replicate.

The IC_{50} is normally the concentration required to inhibit the growth of 50% of the population exposed (SigmaPlot 2015). To determine the IC_{50}, data were plotted in SigmaPlot® software to draw a growth inhibition curve and to calculate IC_{50} using a four parameter logistic equation:

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{EC50}\right)^{-Hillslope}} \qquad (4)$$

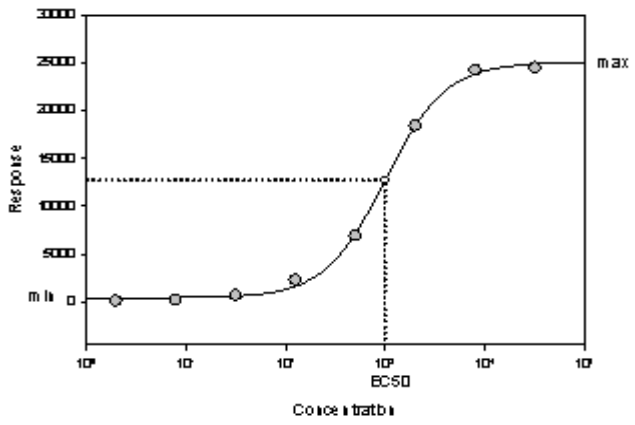


Figure 3.3: Typical concentration-response curve with a variable slope parameter (SigmaPlot 2015)

3.2.10 *Interference of NMs with chlorophyll-extraction assay*

NM toxicity testing towards algae might be challenging, because NMs may interfere with toxicity-testing assays. The aim of the interference experiments was to check whether NMs interact with the assays used to assess Chl a content.

To examine the interference of NMs with the assay used to quantify Chl a concentration, samples were prepared using the same range of concentrations of the test substances in OECD 201 medium in the absence of algal cells to see if there was any interaction with algal biomass measurements. Tests were performed in 250 mL conical glass flasks covered with sponge caps. During the exposure period the incubation temperature, light intensity and shaker speed were exactly the same as in the acute algae growth inhibition tests performed to determinate Ag NM and MWCNT toxicity to *R. subcapitata*. Three replicates of the control (algae in OECD medium), three replicates of OECD medium (just OECD medium in the flasks) and three replicates of each test concentration without algal cells were prepared. Samples for the determination of chlorophyll a were taken from the test flask after 24, 48 and 72 h. Chl a was extracted from samples and measured as described in section 3.2.9.

3.2.11 *Optical density (OD)*

The test is based on the measurement of the optical density of the algal culture in a 10 cm path-length glass cuvette (Hellma Analytics, UK). The UV spectrophotometer (Jenway, model 6715) was blanked using cell-free OECD 201 medium prior to taking the measurement. A volume of 25 ml of algal culture was measured at A_{685} nm. The measured samples were then returned to the original flask. The growth rate and the percentage inhibition of growth were calculated according to OECD 201 (section 3.2.9).

3.2.12 *Interference of tested materials with OD assay*

Interference experiments were carried out spectrophotometrically by determining the optical density of NM samples without algal cells, as described in the interference experiment section above (3.2.10).

3.2.13 *Protein content*

Cultures containing exposed NMs, as well as untreated cultures (control), were grown in batches for 72 h. Cells from each sample were collected by centrifugation (10,000 rpm for 10 min) and re-suspended in 1 mL of 20 mM phosphate buffer (pH 7). Cell

disruption was performed on ice with an ultrasonic probe (LABSONIC) at 10% of power for 5 seconds, followed by a 15-second pause to avoid heat denaturation of proteins. This procedure was repeated 6 times. Extracts were centrifuged (10,000 rpm for 10 min at 4 °C), and the supernatant was collected to be used in a Bradford assay for protein content. Total protein concentration was measured using the Bradford method, with bovine serum albumin (BSA) as the standard protein (Bradford 1976). Briefly, a serial dilution of BSA (1 mg/mL) was prepared as follows: 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 mg/mL of BSA. To measure the protein concentration, a volume of 10 µL of BSA standard, as well as a tested sample, was placed in triplicate in a 96-well white plate. A volume of 200 µL of Bradford reagent (Bradford reagent; cat # B6916, Sigma-Aldrich, UK) was added per well and mixed. The microplate was then placed in the spectrometer with the associated Revelation® software, and the blue colour formed was measured at the wavelength of 570 nm.

3.2.14 *Photosynthesis inhibition (ΦPSII)*

The photosynthetic inhibition of microalgae can be measured using a PHYTO-PAM plankton analyser (Walz, Germany) with the associated PhytoWin® software package (v2.13). The PHYTO-PAM analyser is a pulse amplitude modulation fluorometer which measures Chl fluorescence through excitation at multiple wavelengths using short pulses of light (µs), generated by an array of light-emitting diodes.

Algae were exposed to the test substances (Ag NMs, MWCNTs, $AgNO_3$) for 4 or 24 h as previously described in section 3.2.8. Samples were measured in 1.5 ml volumes in quartz cuvettes (Sigma Aldrich, UK), and stirred using a magnetic stir bar. In order to determine ΦPSII, steady state (F) and maximal (Fm') fluorescence were measured at 470, 645 and 665 nm, and average ΦPSII calculated by taking an average of F and Fm' at the three wavelengths:

$$\Phi PSII = (Fm' - F)/Fm' \qquad (5)$$

ΦPSII inhibition was calculated for each individual treatment replicate, relative to the mean growth rate of the control, using the following equation:

$$\%I_{\Phi PSII} = ((\Phi PSII_{control} - \Phi PSII_{treatment}) / \Phi PSII_{control}) \times 100 \qquad (6)$$

Where: $\%I_{\Phi PSII}$ = percent ΦPSII inhibition; $\Phi PSII_{control}$ = average ΦPSII in the control group and $\Phi PSII_{treatment}$ = average ΦPSII in the treatment replicate.

3.2.15 *Interference of tested materials with PSII activity assay*

The interference of NMs with the photosynthetic activity of *R. subcapitata* measured using a pulse amplitude modulated fluorometer (PHYTO-PAM) was determined for all tested NMs. Three replicates of the control (algae in OECD medium), three replicates of OECD medium (just OECD medium in the flasks) and three replicates of each test concentration without algal cells were prepared. The photosynthetic activity of control samples (algae in medium) and for samples containing only medium and NMs was measured after 4 and 24 h of exposure.

3.2.16 *Statistical analysis*

IC_{50} values were obtained using SigmaPlot® software version 12, and all figures were plotted using GraphPad® Prism 6.0. The significant differences between the IC_{50} values were analysed using one-way or two-way ANOVA, taking $p<0.05$ as significant according to post Tukey's multiple range tests using Minitab® software 18. Each experiment was conducted 3 times. Data are presented as means ± standard deviation of the mean (SD). For the sake of clarity and tidiness, the significant differences signs on the error bars have not been included in some of the graphs.

3.3 Results

3.3.1 *Acute toxicity assessments based on chlorophyll extraction (Chl a)*

R. subcapitata were exposed to bath-/probe-sonicated materials for up to 72 h. Algal growth inhibition was calculated based on the *in vitro* chlorophyll a measurements during the test period up to 72 h post exposure. Algal growth was inhibited in a concentration-dependent manner for $AgNO_3$, Ag NMs and MWCNTs when prepared using bath or probe sonication and was time dependent (Figures 3.4–3.11). In contrast, using both types of dispersion methods with Ag NMs DIS, no growth inhibition was observed (Figures 3.4–3.5). $AgNO_3$ toxicity was enhanced when the solution was prepared using probe sonication, compared to bath sonication. For example, the IC_{50} value for $AgNO_3$ at 72 h was 14.09 (± 2.18) µg/L using bath sonication (Figure 3.4), however, using probe sonication an IC_{50} value of 11.90 (± 0.3) µg/L was obtained (Figure 3.5). However, no statistically significant difference was found between these two IC_{50} values. Furthermore, the toxicity of $AgNO_3$ decreased with time in both types of sonication. For example, the IC_{50} value at 72 h was 14.09 (± 2.18) µg/L, whereas at 24 h it was 7.45 (± 1.25) µg/L using bath sonication. In terms of probe sonication, the

IC_{50} value was 11.90 (± 0.3) µg/L at 72 h, while at 24 h it was 3.63 (± 0.88) µg/L. The obtained results suggested that there was a significant difference between the IC_{50} values of bath and probe sonicated $AgNO_3$ at 24 h.

No significant changes in the toxicity of $AgNO_3$ were observed between 24 and 48 h following preparation by bath-sonication. However, after 72 h of exposure to bath-sonicated $AgNO_3$, a significant decrease in toxicity was observed compared to the other remaining time points. In terms of probe sonication, a significant change in the toxicity of $AgNO_3$ was observed between all tested time points. The results suggest a strong correlation between time of exposure and toxicity of $AgNO_3$ to *R. subcapitata*.

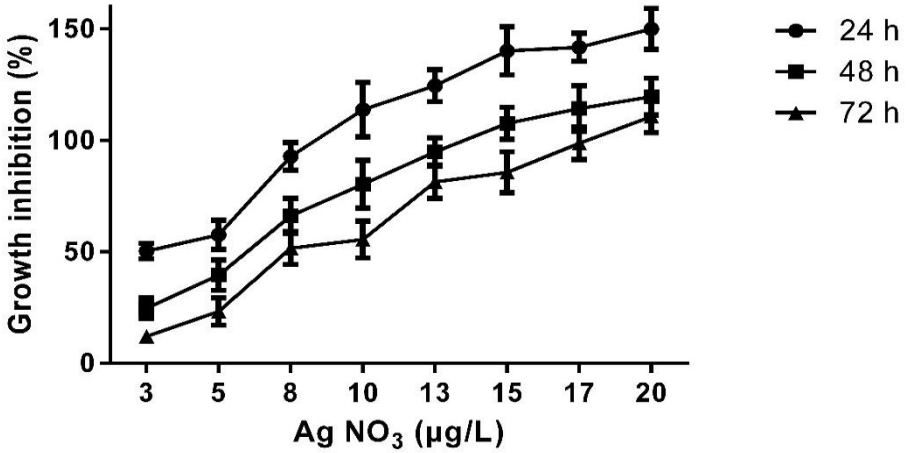


Figure 3.4: Effect of $AgNO_3$ on growth of *R. subcapitata* cultures based on Chl extraction at 24, 48 and 72 h using bath-sonicated $AgNO_3$ (n=3). Algae were exposed to medium (control), or $AgNO_3$ at concentrations of 3 to 20 µg/L for 24, 48 or 72 h. Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean growth inhibition ± SD (standard deviation).

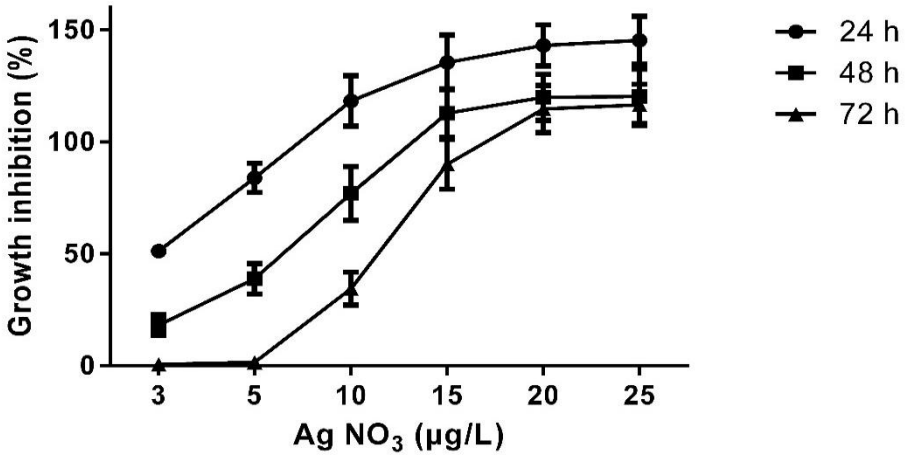


Figure 3.5: Effect of $AgNO_3$ on growth of *R. subcapitata* cultures based on Chl extraction at 24, 48 and 72 h using probe-sonicated $AgNO_3$ (n=3). Algae were exposed to medium (control), or $AgNO_3$ at concentrations of 3 to 25 µg/L for 24, 48 or 72 h. Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean growth inhibition ± SD (standard deviation).

3.3.1.1 Growth-inhibiting effects of Ag NMs on the freshwater microalga *R. subcapitata* based on Chl extraction

The results indicate a concentration response effect, with increased growth inhibition of *R. subcapitata* observed with increasing concentrations of bath- or probe-sonicated Ag NMs at each time point (Figures 3.6–3.7). Ag NMs toxicity was enhanced when the suspension was dispersed using bath sonication, compared with probe sonication. For example, the IC_{50} value at 72 h for Ag NMs was 90.90 (± 25.24) µg/L using bath sonication (Figure 3.6) and 193.76 (± 45.11) µg/L using probe sonication (Figure 3.7). Moreover, these values (90.90 ± 25.24 and 193.76 ± 45.11) were found to be significantly different ($p<0.05$). Furthermore, the toxicity of Ag NMs decreased with tested time points in both types of sonication. For example, the IC_{50} value at 72 h was 90.90 ± 25.24 µg/L, whereas at 24 h it was 18.20 ± 2.37 µg/L using bath sonication. In terms of probe sonication, the IC_{50} value was 193.76 ± 45.11 µg/L at 72 h, while at 24 h it was 53.05 ± 3.69 µg/L. Over time, a significant change in the toxicity of Ag NMs using both types of sonication was observed at all tested time points. Therefore, the results indicate a strong correlation between time of exposure and toxicity of Ag NMs to *R. subcapitata*.

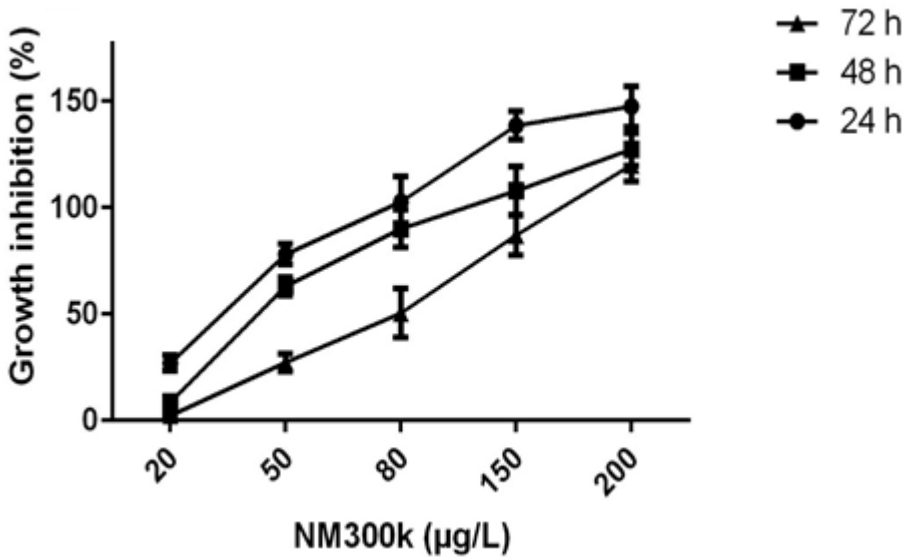


Figure 3.6: Effect of Ag NMs on growth of *R. subcapitata* cultures based on Chl extraction at 24, 48 and 72 h using bath-sonicated Ag NMs (n=3). Algae were exposed to medium (control), or Ag NMs at concentrations of 20 to 200 µg/L for 24, 48 or 72 h. Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean growth inhibition ± SD (standard deviation).

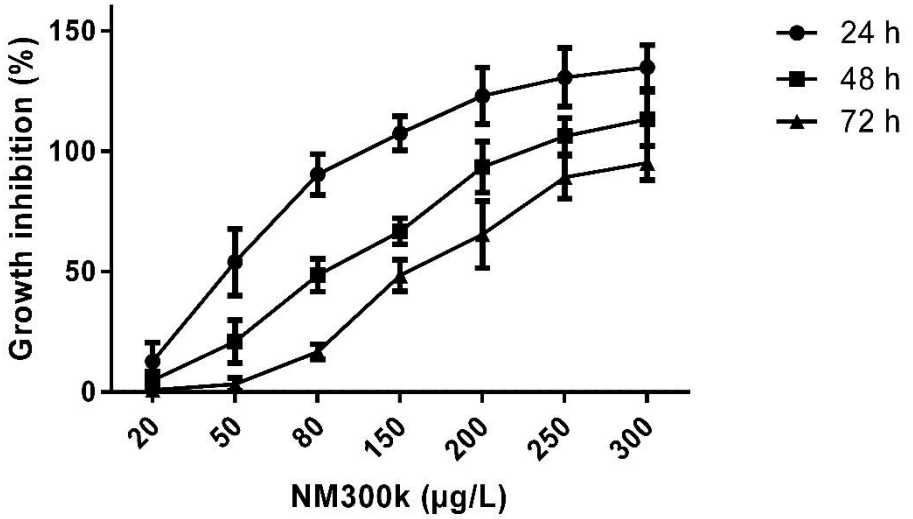


Figure 3.7: Effect of Ag NMs on growth of *R. subcapitata* cultures based on Chl extraction at 24, 48 and 72 h using probe-sonicated Ag NMs (n=3). Algae were exposed to medium (control), or Ag NMs at concentrations of 20 to 300 µg/L for 24, 48 or 72 h. Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean growth inhibition ± SD (standard deviation).

3.3.1.2 Growth-inhibiting effects of Ag NM dispersant (NM300k DIS) on the freshwater microalga *R. subcapitata* based on Chl extraction

There is no noticeable negative effect from exposure of *R. subcapitata* to NM300k DIS over the course of 72 h using the two different sonication methods (Figures 3.8 and 3.9).

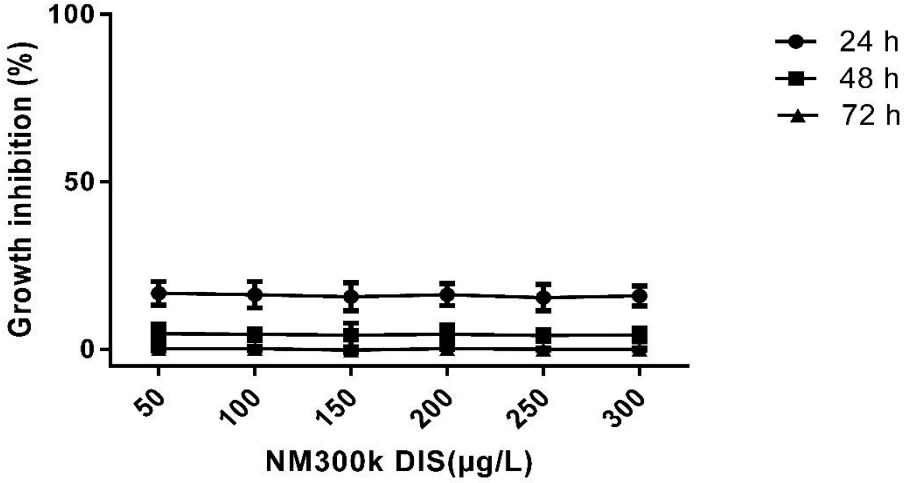


Figure 3.8: Effect of NM300k DIS on growth of *R. subcapitata* cultures based on Chl extraction at 24, 48 and 72 h using bath-sonicated NM300k DIS (n=3). Algae were exposed to medium (control), or NM300k DIS at concentrations of 50 to 300 µg/L for 24, 48 or 72 h. Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean growth inhibition ± SD (standard deviation).

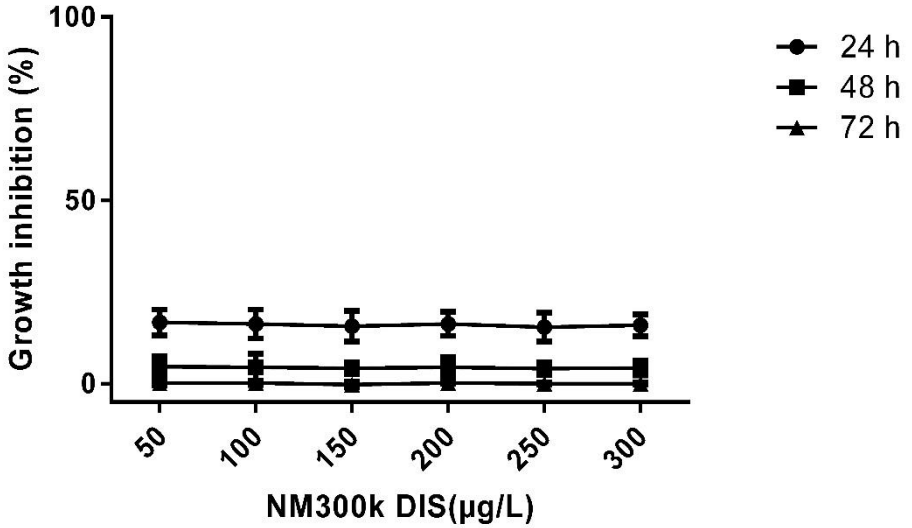


Figure 3.9: Effect of NM300k DIS on growth of *R. subcapitata* cultures based on Chl extraction at 24, 48 and 72 h using probe-sonicated NM300k DIS (n=3). Algae were exposed to medium (control), or NM300k DIS at concentrations of 50 to 300 µg/L for 24, 48 or 72 h. Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean growth inhibition ± SD (standard deviation).

3.3.1.3 Growth-inhibiting effects of MWCNTs on the freshwater microalga *R. subcapitata* based on Chl extraction

The results indicate a concentration-dependent effect for MWCNTs, with increased growth inhibition of *R. subcapitata* observed with increasing MWCNT concentration at each time point (Figures 3.10 and 3.11). MWCNT toxicity was enhanced when the suspension was dispersed using probe sonication, as opposed to bath sonication. The obtained IC_{50} value at 72 h for MWCNTs was 28.59 ± 7.59 mg/L using bath sonication (Figure 3.10) and 6.83 ± 0.34 mg/L using probe sonication (Figure 3.11). Moreover, these values (6.83 ± 0.34 and 28.59 ± 7.59 mg/L) are significantly different ($p<0.05$). IC_{50} values obtained for *R. subcapitata* after exposure to MWCNT using the probe- and bath-sonication processes are significantly different to each other at all 3 exposure timepoints. No significant changes in IC_{50} values were observed between two timepoints (24 and 48 h) of exposure to MWCNTs dispersed using bath sonication. However, a significant decrease in the toxicity was observed at the last timepoint (72 h) compared to the previous timepoints (24 and 48 h) for both sonication methods. In contrast, there is a significant difference between the two methods of sonication.

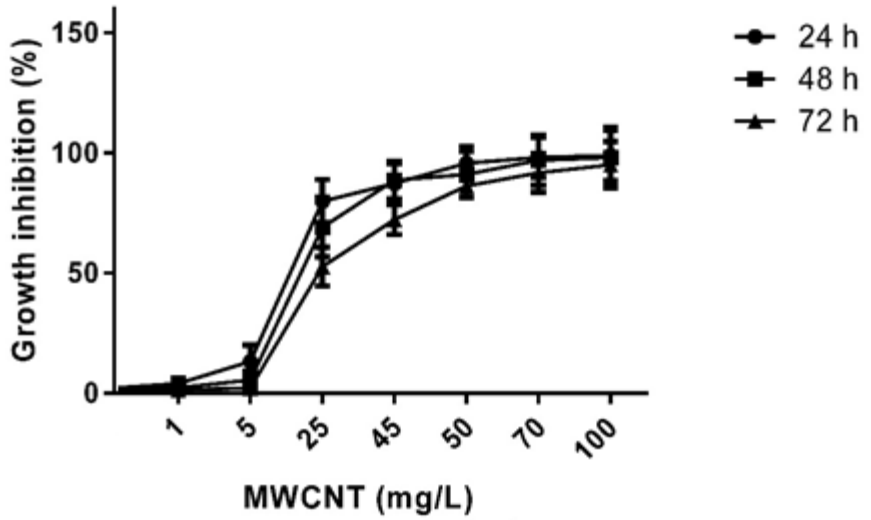


Figure 3.10: Effect of MWCNTs on growth of *R. subcapitata* cultures based on Chl extraction at 24, 48 and 72 h using bath-sonicated MWCNTs (n=3). Algae were exposed to medium (control), or MWCNTs at concentrations of 1 to 100 mg/L for 24, 48 or 72 h. Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean growth inhibition ± SD (standard deviation).

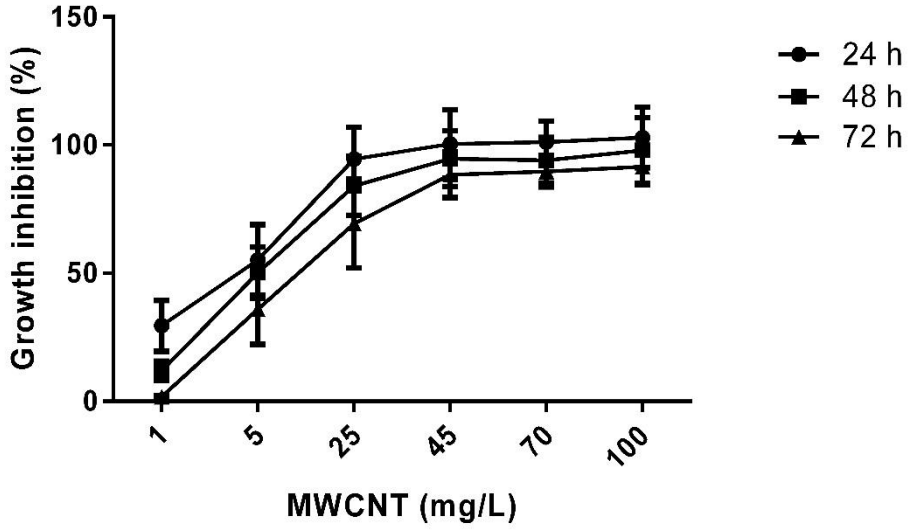


Figure 3.11: Effect of MWCNTs on growth of *R. subcapitata* cultures based on Chl extraction at 24, 48 and 72 h using probe-sonicated MWCNTs (n=3). Algae were exposed to medium (control), or MWCNTs at concentrations of 1 to 100 mg/L for 24, 48 or 72 h. Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean growth inhibition ± SD (standard deviation).

A summary of the IC_{50} values (72 h) obtained for *R. subcapitata* exposed to $AgNO_3$, Ag NMs, NM300k DIS and MWCNTs based on chlorophyll extraction is presented in Table 3.1. Based on the IC_{50} values the toxicity of the test substances can be ranked: $AgNO_3$ > Ag NMs > MWCNTs. In terms of sonication methods, the toxicity of $AgNO_3$ and MWCNTs was enhanced when probe sonication was used (although not statistically significant for $AgNO_3$). In contrast, the toxicity of Ag NMs was enhanced when bath sonication was used.

Table 3.1: IC_{50} values (72 h) obtained for *R. subcapitata* exposed to $AgNO_3$, Ag NMs, NM300k DIS and MWCNTs, based on chlorophyll extraction. Data are mean ± SD (standard deviation).

Test material	Sonication method	
	IC_{50} values with bath (µg/L)	IC_{50} values with probe (µg/L)
$AgNO_3$	14.09 (± 2.18)	11.90 (± 0.3)
Ag NMs (NM300k)	90.90 (± 25.24)	193.76 (± 45.11)
Ag NMs DIS (NM300K DIS)	ND	ND
MWCNT (NM400)	28,590 (± 759)	6,830 (± 343)

Based on the results of two-way ANOVA, the interaction of IC_{50} values for both type of NMs (Ag NMs and MWCNTs) were significantly affected by the interaction of the two factors, time points and sonication type (Table 3.2).

Table 3.2: Results of two-way ANOVA interaction results for IC_{50} values for both type of NMs (Ag NMs and MWCNTs) for 72h

NMs	Source	DF	Adj MS	F-Value	*P*-Value
Ag NMs	Sonication type	5	11459.2	1191.61	0.031
Ag NMs	Time	5	11459.2	1191.61	0.001
Ag NMs	Sonication type*time	2	1887.3	196.25	0.021
MWCNTs	Sonication type	5	11459.2	1191.61	0.001
MWCNTs	Time	5	2589.39	26.45	0.018
MWCNTs	Sonication type*time	2	9509.9	31.6	0.001

3.3.2 *Interference of tested materials with Chl extraction assay*

3.3.2.1 Interference of $AgNO_3$ with Chl extraction assay

A test to ascertain the potential interference of tested materials with the Chl extraction assay using a fluorometer was performed (Figures 3.12–3.17). Chl extractions were taken from all test substances in the absence of algal cells to identify whether the materials interfered with the Chl measurements. Alongside this, a control sample with algal cells was run as a positive control (with no tested material) and another sample was used as a negative control (just OECD 201 medium without algal cells).

OD values for the algal controls were 0.15, 1.00 and 2.45 A_{685nm}, at 24, 48 and 72 h, respectively. The observed OD values for algal controls are much higher than, and significantly different from, those observed for the $AgNO_3$ alone at all tested time points. The potential interference of $AgNO_3$ suspensions prepared using bath and probe sonication with chlorophyll extracted is shown in Figures 3.12 and 3.13, respectively. No interference of OECD 201 medium and $AgNO_3$ was observed at 3 different experimental timepoints across all tested concentrations of $AgNO_3$.

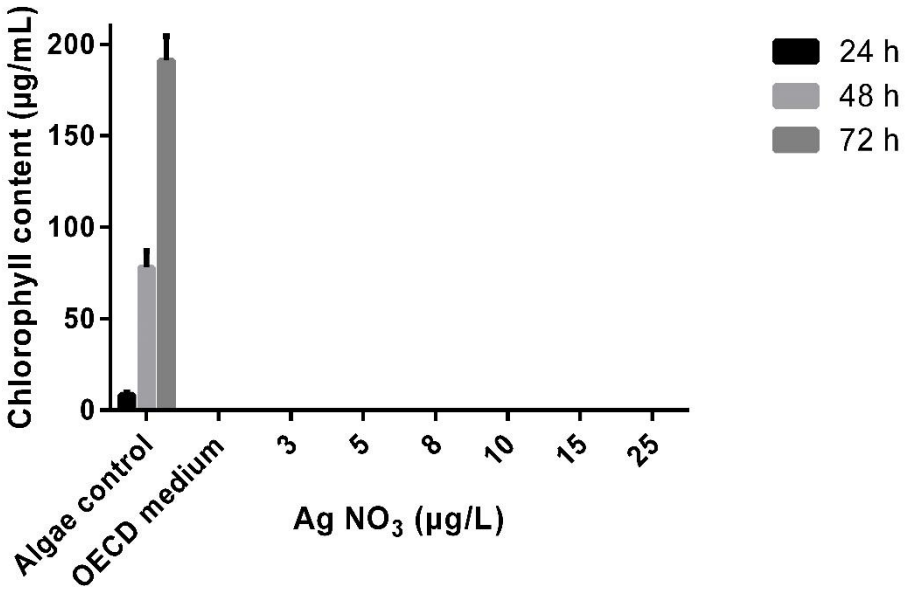


Figure 3.12: Interference of $AgNO_3$ with Chl extraction assay using bath-sonicated $AgNO_3$ (n=3). Treatments were algae were exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or $AgNO_3$ at concentrations of 3 to 20 µg/L for 24, 48 or 72 h (without algal cells). Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean chlorophyll content ± SD (standard deviation).

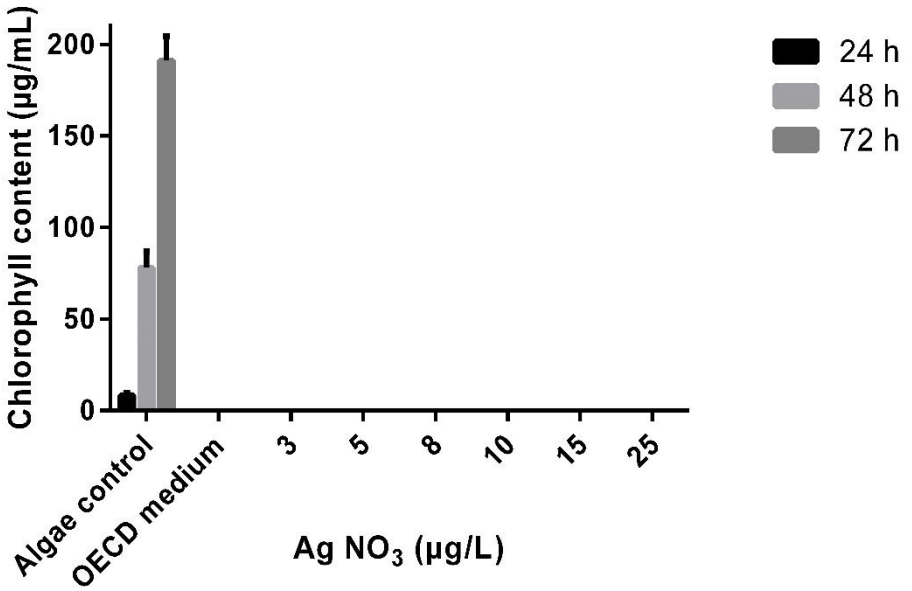


Figure 3.13: Interference of $AgNO_3$ with Chl extraction assay using probe-sonicated $AgNO_3$ (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or $AgNO_3$ at concentrations of 3 to 25 µg/L for 24, 48 or 72 h (without algal cells). Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean chlorophyll content ± SD (standard deviation).

3.3.2.2 Interference of Ag NMs with Chlorophyll extraction assay

The potential interference with chlorophyll extraction of Ag NM suspensions prepared using both sonication processes is shown in Figures 3.14 and 3.15 for bath and probe sonication, respectively. No interference of OECD medium and Ag NMs was observed at 3 different experimental timepoints across all tested concentrations of NM300k.

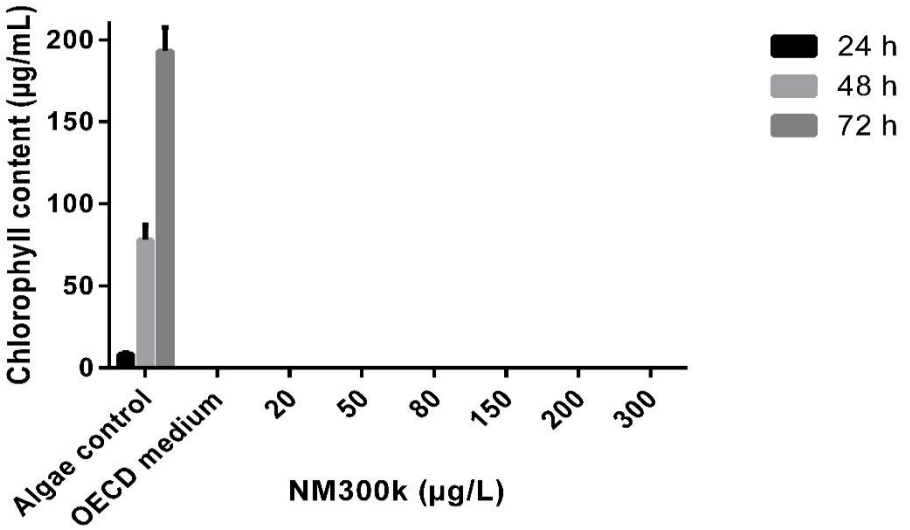


Figure 3.14: Interference of Ag NMs with Chl extraction assay using bath-sonicated Ag NMs (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or Ag NMs at concentrations of 20 to 300 µg/L for 24, 48 or 72 h (without algal cells). Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean chlorophyll content ± SD (standard deviation).

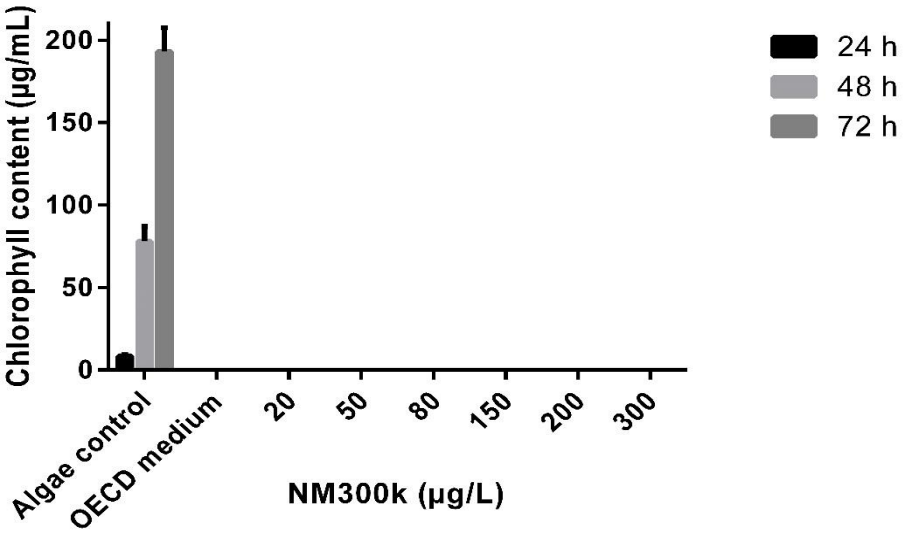


Figure 3.15: Interference of Ag NMs with Chl extraction assay using probe-sonicated Ag NMs (n=3). Treatments were algae were exposed to OECD 201 medium (+ control), or OECD 201 medium (- control without algal cells) or Ag NMs at concentrations of 20 to 300 µg/L for 24, 48 or 72 h (without algal cells). Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean chlorophyll content ± SD (standard deviation).

3.3.2.3 Interference of MWCNTs with Chlorophyll extraction assay

The potential interference with chlorophyll extraction of MWCNT suspensions prepared using bath sonication is shown in Figures 3.16 and 3.17 for bath and probe sonication, respectively. No interference of OECD medium and MWCNTs was observed at 3 different experimental timepoints across all tested concentrations of MWCNTs.

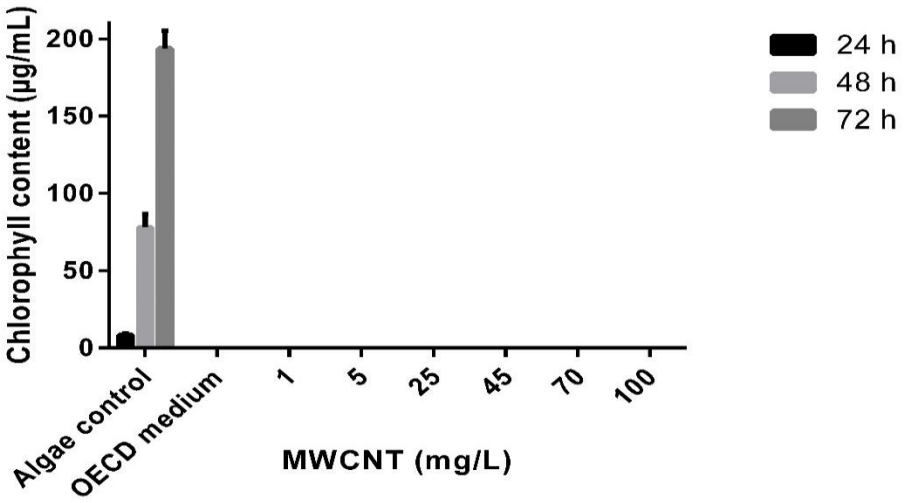


Figure 3.16: Interference of MWCNTs with Chl extraction assay using bath-sonicated MWCNTs (n=3). Treatments were algae were exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or MWCNTs at concentrations of 1 to 100 mg/L for 24, 48 or 72 h (without algal cells). Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean chlorophyll content ± SD (standard deviation).

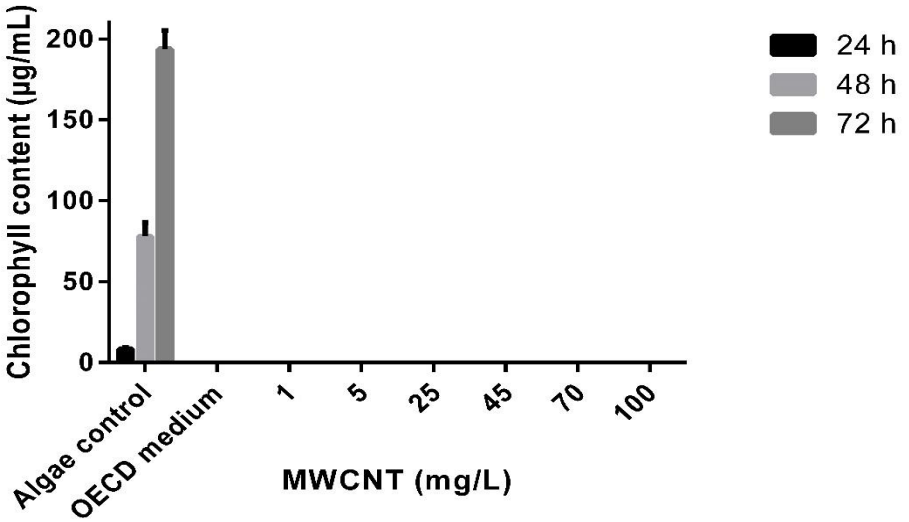


Figure 3.17: Interference of MWCNTs with Chl extraction assay using probe-sonicated MWCNTs (n=3). Treatments were algae were exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or MWCNTs at concentrations of 1 to 100 mg/L for 24, 48 or 72 h (without algal cells). Chlorophyll was then extracted and measured using fluorimetry. Data are expressed as mean chlorophyll content ± SD (standard deviation).

Overall, when using Chl extraction methods to assess the toxicity of tested materials, there is no noticeable interference from the used materials on the Chl extraction assay.

3.3.3 *Acute toxicity assessments based on optical density*

3.3.3.1 The growth inhibition of *R. subcapitata* exposed to $AgNO_3$ quantified based on OD

A concentration and time-dependent growth inhibition was observed for Ag NMs, $AgNO_3$ and MWCNTs (Figures 3.18–3.23). $AgNO_3$ stimulated a concentration-dependent decrease in algal growth, with increasing concentrations stimulating increased toxicity at each time point. The toxicity of $AgNO_3$ was enhanced when prepared using probe sonication. For example, the IC_{50} value at 72 h was 14.78 µg Ag/L after 72 h of exposure to bath-sonicated suspensions of $AgNO_3$, whereas the same growth inhibition of 50% was reached at 12.05 µg Ag/L of probe-sonicated $AgNO_3$ (Figures 3.18–3.19). However, these values (14.78 and 12.05) are not significantly different (p<0.05).

Furthermore, the toxicity of $AgNO_3$ decreased at the tested time points with both types of sonication. For example, the IC_{50} value at 72 h was 14.78 µg/L, whereas at 24 h it was 9.5 µg/L using bath sonication. In terms of probe sonication, the IC_{50} value was 12.05 µg/L at 72 h, while at 24 h it was 3.63 µg/L.

No significant changes in the toxicity of $AgNO_3$ were observed after all tested time points following preparation by bath sonication. In contrast, a significant change in the toxicity of $AgNO_3$ was observed after 72 h compared to 24 and 48 h following preparation by probe-sonication. The results suggest a strong correlation between time of exposure and toxicity of $AgNO_3$ to *R. subcapitata*. Generally, probe-sonicated $AgNO_3$ increased the growth inhibition of *R. subcapitata* more than bath-sonicated $AgNO_3$ suspensions.

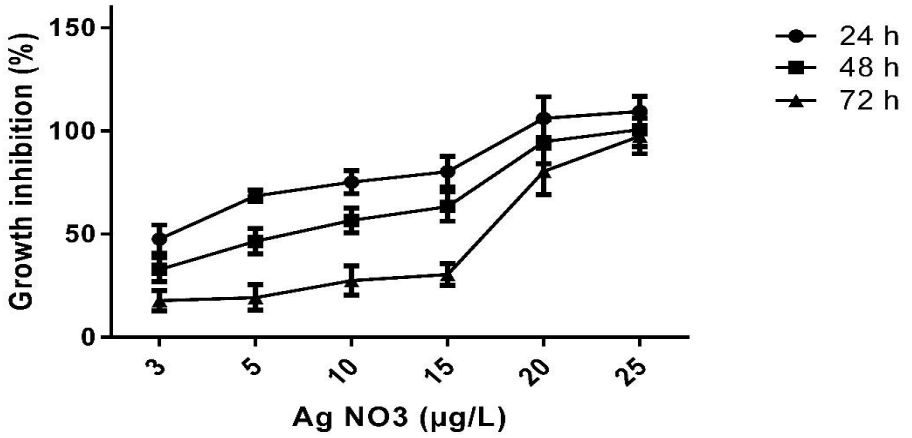


Figure 3.18: Effect of $AgNO_3$ on growth of *R. subcapitata* cultures based on optical density at 24, 48 and 72 h using bath-sonicated $AgNO_3$ (n=3). Algae were exposed to OECD 201 medium (control), or $AgNO_3$ at concentrations of 3 to 20 µg/L for 24, 48 or 72 h. Optical density was then measured using a spectrophotometer. Data are expressed as mean growth inhibition ± SD (standard deviation).

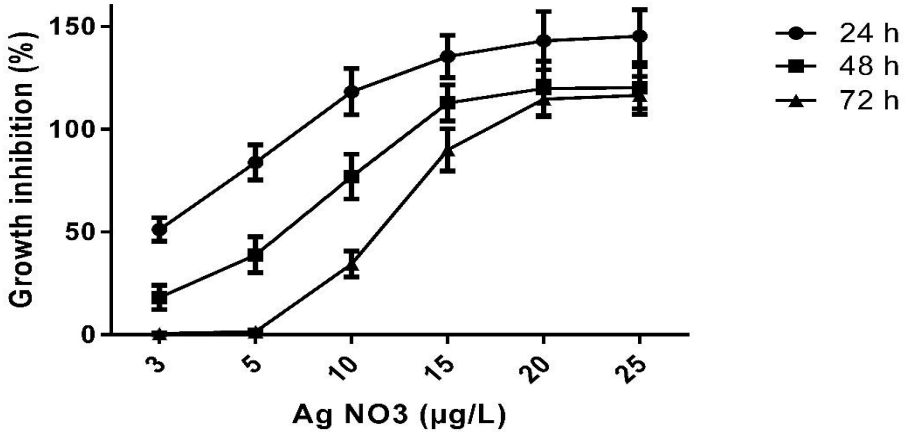


Figure 3.19: Effect of $AgNO_3$ on growth of *R. subcapitata* cultures based on optical density at 24, 48 and 72 hrs using probe-sonicated $AgNO_3$ (n=3). Algae were exposed to OECD 201 medium (control), or $AgNO_3$ at concentrations of 3 to 20 µg/L for 24, 48 or 72 h. Optical density was then measured using a spectrophotometer. Data are expressed as mean growth inhibition ± SD (standard deviation).

3.3.3.2 The growth inhibition of *R. subcapitata* exposed to Ag NMs quantified based on OD

Ag NMs stimulated a time and concentration dependent effect on algal growth (Figures 3.20–3.21). An increasing concentration of Ag NMs stimulated a greater toxic effect at each time point. NMs were more toxic when prepared using bath sonication. For example, the IC_{50} value at 72 h for Ag NMs was 88.01 µg/L using bath sonication (Figure 3.20), however, using probe sonication an IC_{50} value of 175.15

µg/L was obtained (Figure 3.21). Moreover, these values – 88.1 and 175.15 – are significantly different (p<0.05). A significant decrease in the toxicity of Ag NMs was observed for all tested time points following preparation by bath and probe sonication methods.

Strong time-response and concentration-response relationship was observed for both bath- and probe-sonicated Ag NM suspensions. Bath-sonicated suspensions affected the growth inhibition more strongly than probe-sonicated suspensions. The opposite effect of the sonication type on growth inhibition was observed for $AgNO_3$ exposures.

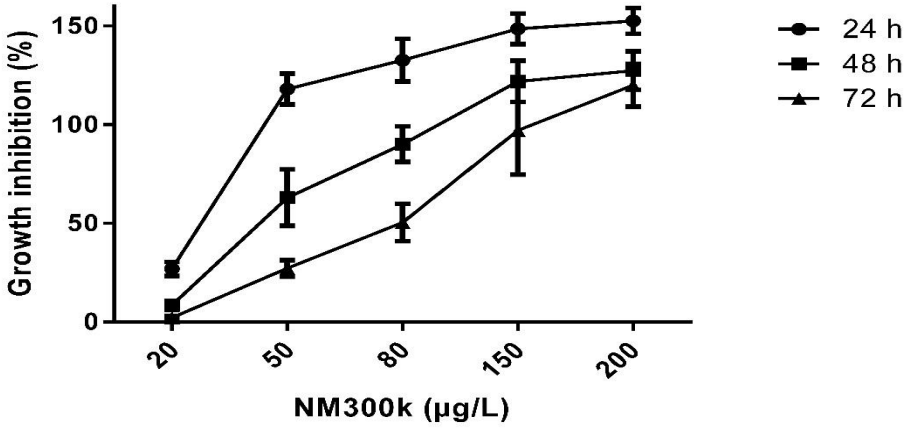


Figure 3.20: Effect of Ag NMs on growth of *R. subcapitata* cultures based on optical density at 24, 48 and 72 h using bath-sonicated Ag NMs (n=3). Algae were exposed to OECD 201 medium (control), or Ag NMs at concentrations of 20 to 200 µg/L for 24, 48 or 72 h. Optical density was then measured using a spectrophotometer. Data are expressed as mean growth inhibition ± SD (standard deviation).

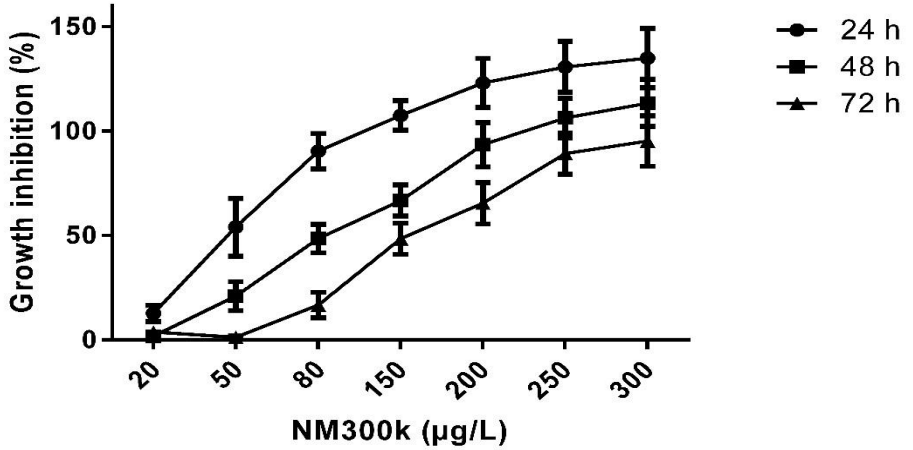


Figure 3.21: Effect of Ag NMs on growth of *R. subcapitata* cultures based on optical density at 24, 48 and 72 h using probe-sonicated Ag NMs (n=3). Algae were exposed to OECD 201 medium (control), or Ag NMs at concentrations of 20 to 300 µg/L for 24, 48 or 72 h. Optical density was then measured using a spectrophotometer. Data are expressed as mean growth inhibition ± SD (standard deviation).

3.3.3.3 The growth inhibition of *R. subcapitata* exposed to MWCNTs quantified based on OD

MWCNTs caused a time- and concentration- dependent effect on algal growth (Figures 3.22–3.23). Increasing concentrations of MWCNTs caused an increase in algal growth inhibition at each time point. Based on OD, bath-sonicated MWCNTs were more toxic than probe-sonicated MWCNTs. For example, the IC_{50} for bath-sonicated MWCNTs was 40.28 mg/L after 72 h, while it was 103.76 mg/L for probe-sonicated MWCNTs. Moreover, these values (40.28 and 103.76) are significantly different ($p<0.05$). Furthermore, the toxicity of MWCNTs decreased with tested time points in both types of sonication. For example, the IC_{50} value at 72 h was 40.28 mg/L, whereas at 24 h it was 12.20 mg/L using bath sonication. In terms of probe-sonicated MWCNTs, the IC_{50} value was 103.76 mg/L at 72 h, while at 24 h it was 64.73 mg/L. No significant changes in the toxicity of MWCNTs were observed after 24 and 48 h following preparation of MWCNTs by bath and probe sonication methods. However, after 72 h of exposure to bath- and probe-sonicated MWCNTs, a significant increase in toxicity was observed compared to the other timepoints.

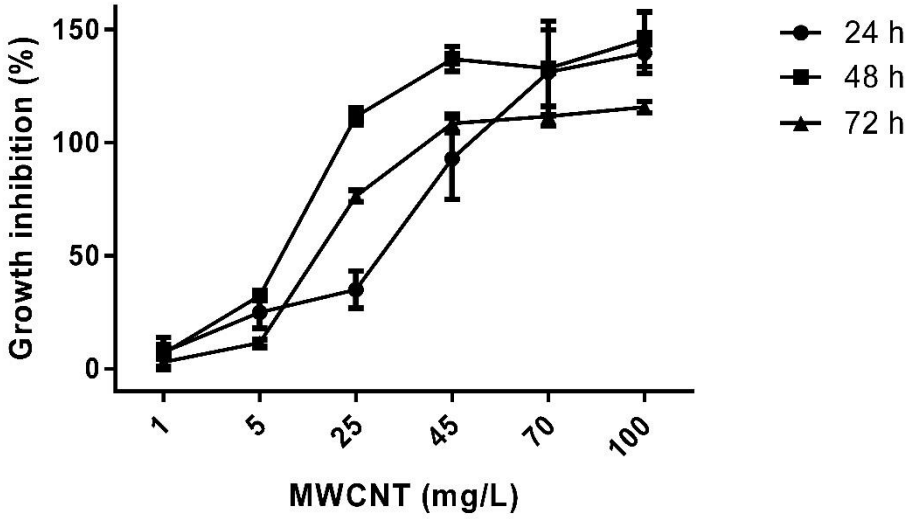


Figure 3.22: Effect of MWCNTs on growth of *R. subcapitata* cultures based on optical density at 24, 48 and 72 h using bath-sonicated MWCNTs (n=3). Algae were exposed to OECD 201 medium (control), or MWCNTs at concentrations of 1 to 100 mg/L for 24, 48 or 72 h. Optical density was then measured using a spectrophotometer. Data are expressed as mean growth inhibition ± SD (standard deviation).

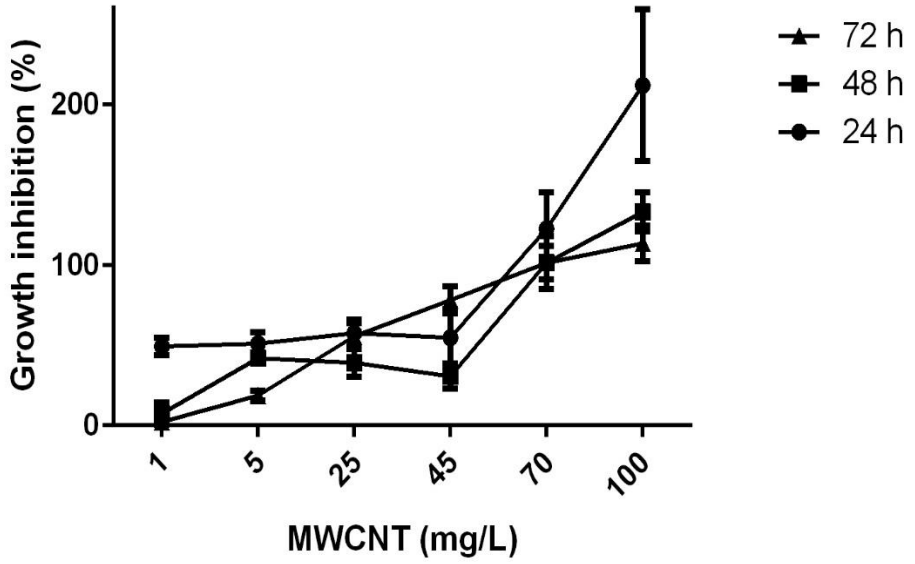


Figure 3.23: Effect of MWCNTs on growth of *R. subcapitata* cultures based on optical density at 24, 48 and 72 h using probe-sonicated MWCNTs (n=3). Algae were exposed to OECD 201 medium (control), or MWCNTs at concentrations of 1 to 100 mg/L for 24, 48 or 72 h. Optical density was then measured using a spectrophotometer. Data are expressed as mean growth inhibition ± SD (standard deviation).

A summary of the IC_{50} values (72 h) obtained for $AgNO_3$, Ag NMs and MWCNTs exposed to *R. subcapitata* based on optical density is presented in Table 3.3. Based on the IC_{50} values obtained by optical density, the toxicity ranking was as follows: $AgNO_3$ > Ag NMs > MWCNTs. Across all tested suspensions, the highest toxic effect was observed for $AgNO_3$ treatment. In terms of sonication methods, the toxicity of $AgNO_3$ was enhanced when probe sonication was used, although not statistically significant. However, the toxicity of Ag NMs and MWCNTs was enhanced when bath sonication was used.

Table 3.3: IC_{50} values (72 h) obtained for *R. subcapitata* exposed to $AgNO_3$, Ag NMs and MWCNTs based on optical density. Data are mean ± SD (standard deviation).

Test material	Sonication method	
	IC_{50} values with bath (µg/L)	**IC_{50} values with probe (µg/L)**
$AgNO_3$	14.78 (± 1.84)	12.05 (± 1.22)
Ag NMs (NM300k)	88.06 (± 11.08)	175.17 (± 13.3)
MWCNT (NM400)	40,280 (± 13,880)	103,760 (± 10,230)

3.3.4 *Interference of tested materials with optical density assay*

3.3.4.1 Interference of $AgNO_3$ with optical density assay

A test to ascertain the potential interference of tested materials with the optical density assay using a spectrophotometer was performed (Figures 3.24–3.29). Optical density measurements were made for all test substances in the absence of algal cells to identify whether they contributed to the OD readings. Moreover, a control sample with algal cells was run as a positive control (with no tested material) and another sample was used as a negative control (just OECD 201 medium without algal cells).

The potential interference of $AgNO_3$ with the optical density assay is presented in the figures below (Figures 3.24–3.25). No OD measurements could be made for any of the concentrations of silver nitrate, suggesting that there was no interference of $AgNO_3$ using either type of sonication at 3 different experimental timepoints.

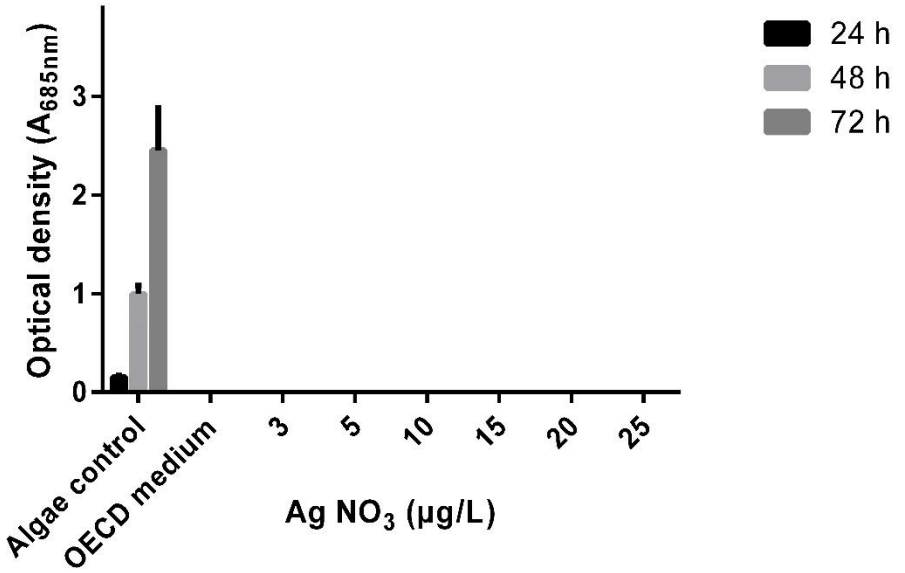


Figure 3.24: Interference of $AgNO_3$ with optical density assay using bath-sonicated $AgNO_3$ (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or $AgNO_3$ at concentrations of 3 to 20 µg/L for 24, 48 or 72 h (without algal cells). Optical density was then measured using a spectrophotometer. Data are expressed as mean optical density ± SD (standard deviation).

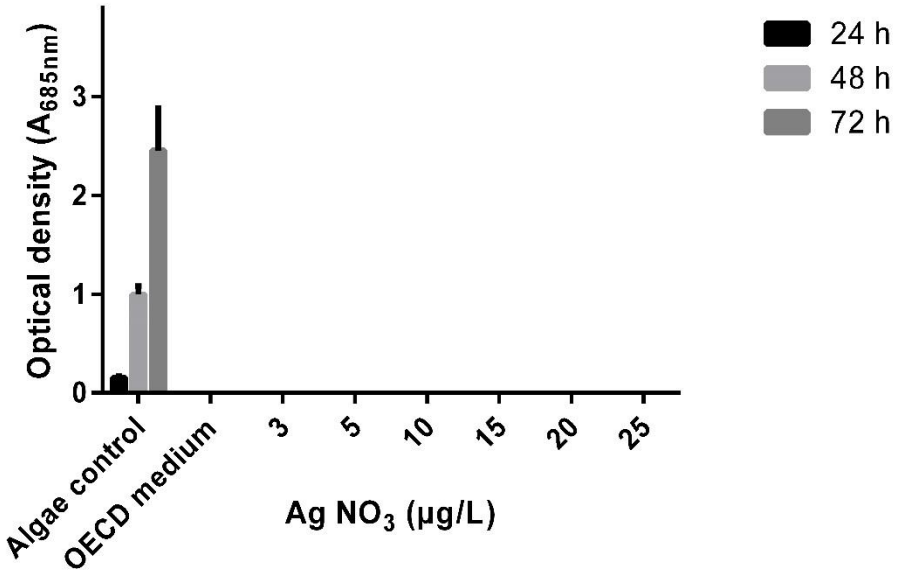


Figure 3.25: Interference of $AgNO_3$ with optical density assay using probe-sonicated $AgNO_3$ (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or $AgNO_3$ at concentrations of 3 to 25 µg/L for 24, 48 or 72 h (without algal cells). Optical density was then measured using a spectrophotometer. Data are expressed as mean optical density ± SD (standard deviation).

3.3.4.2 Interference of Ag NMs with optical density assay

The potential interference of Ag NMs with the OD assay is shown in the figures below (Figures 3.26–3.27). OD values for the algae controls were 0.15, 1.00 and 2.45 A_{685nm}, at 24, 48 and 72 h, respectively. The observed OD values for algal controls are much higher and significantly different from those observed for the Ag NMs alone at all tested time points.

Bath-sonicated Ag NMs (in the absence of algae) showed a slight but not significant increase in optical density. No time-dependent effect was observed, with no significant change in OD measurements observed at any of the tested time points.

Moreover, probe-sonicated Ag NMs suspensions did not show any interference with the optical density assay at the concentrations tested (20–200 µg/L), with the exception of the highest concentration (300 µg/L) at 24 h, when a significant increase in OD was observed compared to OECD medium (in the absence of algae).

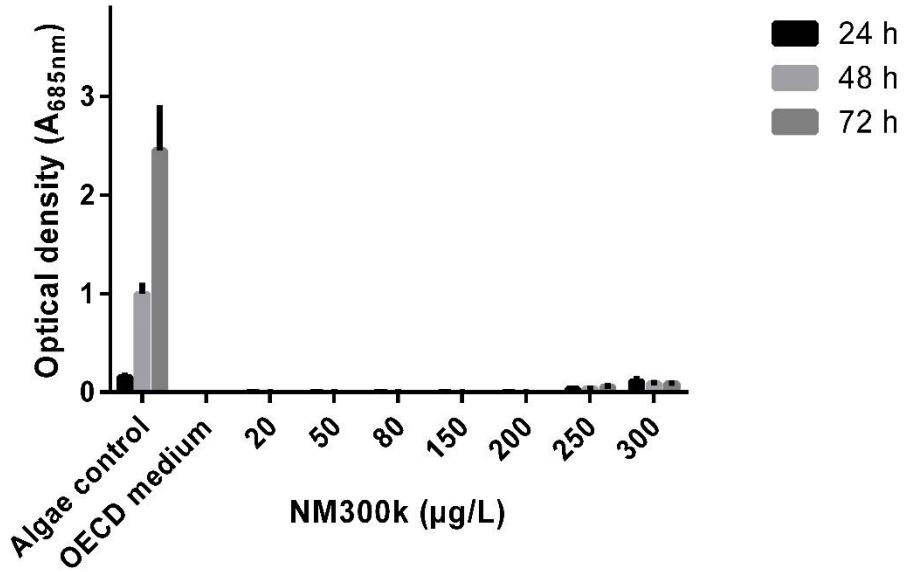


Figure 3.26: Interference of Ag NMs with optical density assay using bath-sonicated Ag NMs (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or Ag NMs at concentrations of 20 to 300 µg/L for 24, 48 or 72 h (without algal cells). Optical density was then measured using a spectrophotometer. Data are expressed as mean optical density ± SD (standard deviation).

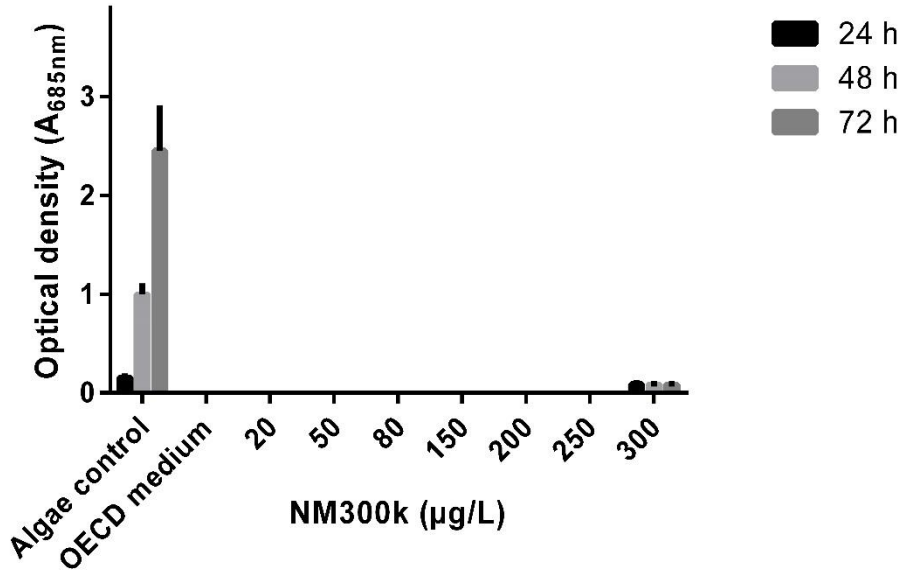


Figure 3.27: Interference of Ag NMs with optical density assay using probe-sonicated Ag NMs (n=3). Algae were exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or Ag NMs at concentrations of 20 to 300 µg/L for 24, 48 or 72 h (without algal cells). Optical density was then measured using a spectrophotometer. Data are expressed as mean optical density ± SD (standard deviation).

3.3.4.3 Interference of MWCNTs with optical density assay

The potential interference of bath- and probe-sonicated MWCNTs is shown in the figures below (Figures 3.28–3.29). OD values for the algae controls were 0.15, 1.00 and 2.45 A_{685nm}, at 24, 48 and 72 h, respectively. A concentration-dependent increase in OD values was observed for bath- and probe-sonicated MWCNTs. At 72 h, the increase in OD was significant for all concentrations compared to the negative control, with the exception of the lowest two concentrations (1 and 5 mg/L) of MWCNTs. In contrast, there were no significant differences at the highest tested bath-sonicated MWCNT concentration (100 mg/L) as 2.36 A_{685nm} was observed compared to an algal-positive control result of 2.19 A_{685nm} at 72 h. Furthermore, in terms of probe sonication, there was a higher increase in OD of MWCNTs compared to the positive control. The obtained OD for 100 mg/L MWCNTs at 72 h was 3.3 A_{685nm}. Overall, MWCNTs significantly interfere with the OD assay, therefore this assay cannot be used for *R. subcapitata* growth inhibition assays.

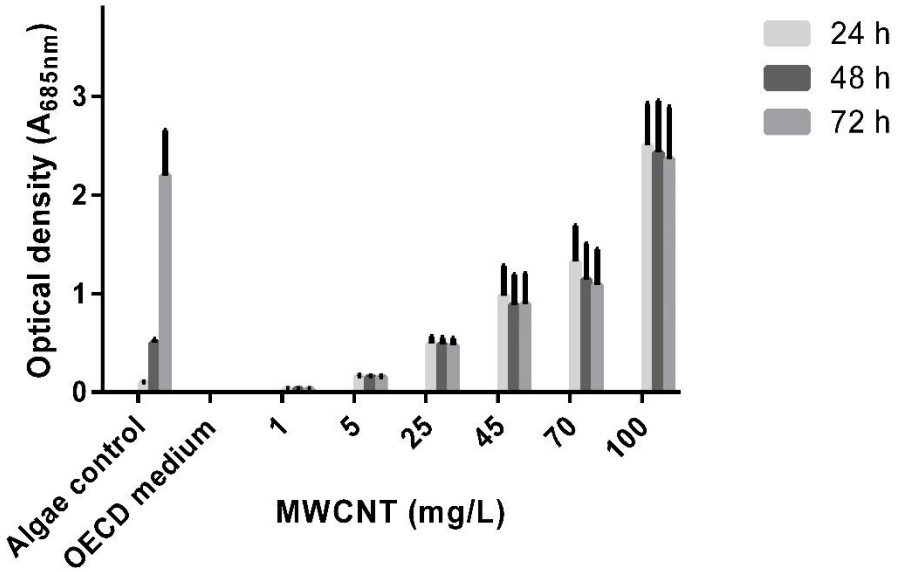


Figure 3.28: Interference of MWCNTs with optical density assay using bath-sonicated MWCNTs (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or MWCNTs at concentrations of 1 to 100 mg/L for 24, 48 or 72 h (without algal cells). Optical density was then measured using a spectrophotometer. Data are expressed as mean optical density ± SD (standard deviation).

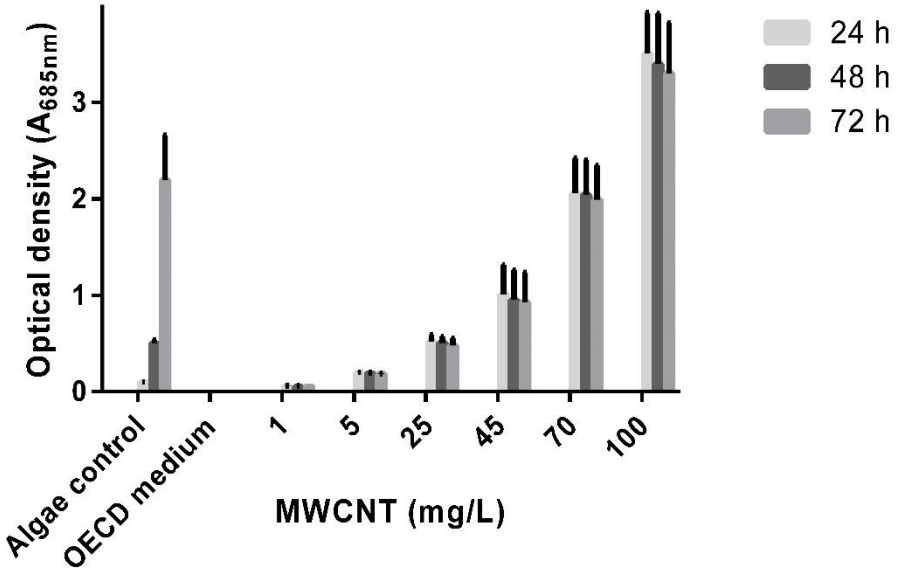


Figure 3.29: Interference of MWCNTs with optical density assay using probe-sonicated MWCNTs (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or MWCNTs at concentrations of 1 to 100 mg/L for 24, 48 or 72 h (without algal cells). Optical density was then measured using a spectrophotometer. Data are expressed as mean optical density ± SD (standard deviation).

Overall, after using optical density methods to assess the toxicity of tested materials, there is no noticeable interference from the $AgNO_3$ used in optical density assays. However, there is interference from the NMs used (Ag NMs and MWCNTs) in optical density assays, particularly the MWCNTs, due to their morphology, but more importantly, due to the concentration used.

3.3.5 *Acute toxicity assessments based on protein content*

3.3.5.1 The growth inhibition of *R. subcapitata* exposed to Ag NMs quantified based on protein content

R. subcapitata was exposed to bath- and probe-sonicated Ag NMs at concentrations ranging from 20 to 80 and 20 to 150 µg/L, respectively, for 72 h. Ag NMs caused a concentration-dependent decrease in protein concentration. The protein content on the control samples of *R. subcapitata* was 0.26 (± 0.04) mg/mL. The two lowest concentrations of bath-sonicated Ag NMs (20 and 50 µg/L) did not reduce protein content significantly in exposed algal cells. A significant decrease in protein content was observed at concentrations of 80 µg/L of bath-sonicated Ag NMs, compared to the control samples. The protein content of *R. subcapitata* exposed to 80 µg/L of bath-sonicated Ag NMs was 0.105 (± 0.023) mg/mL.

In terms of probe sonication, the lowest concentration of Ag NMs (20 µg/L) significantly increased protein content, compared to the control samples. The protein content of algae exposed to probe-sonicated Ag NMs at a concentration of 50 µg/L was 0.27 (± 0.04) mg/mL. At the two highest concentrations tested (80 and 150 µg/L), the protein content of the algae was significantly lower than in the control samples. The protein content of *R. subcapitata* exposed to 150 µg/L of probe-sonicated Ag NMs was 0.06 (± 0.02) mg/mL.

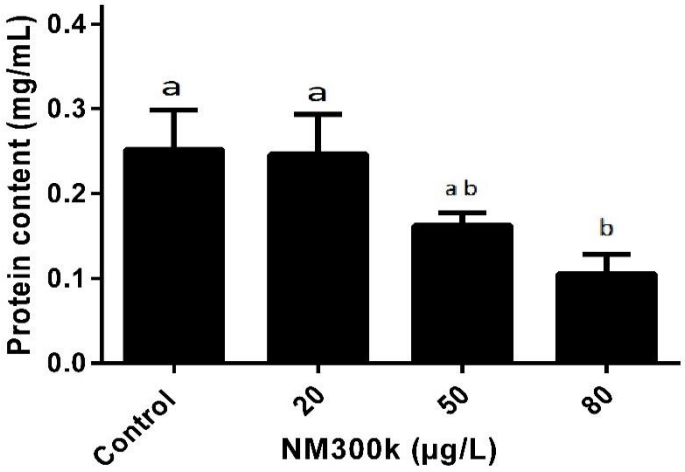


Figure 3.30: Effect of Ag NMs on growth of *R. subcapitata* cultures based on protein content at 72 h using bath-sonicated Ag NMs (n=3). Algae were exposed to OECD 201 medium (control), or Ag NMs at concentrations of 20, 50 and 80 µg/L for 72 h. Protein content was then measured using a Bradford assay. Data are expressed as protein content ± SD (standard deviation).

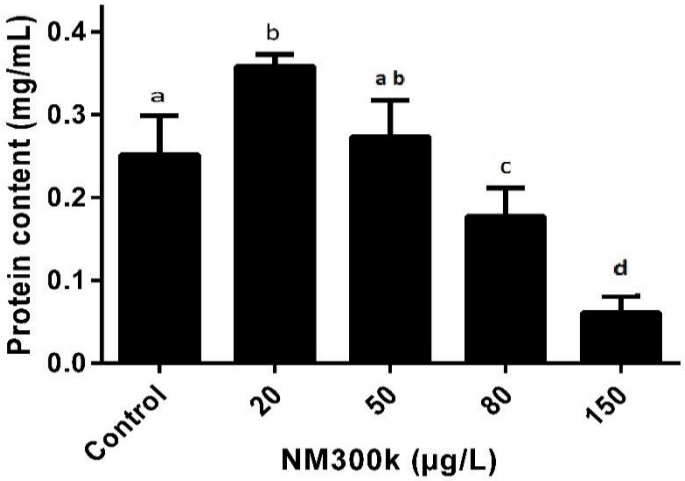


Figure 3.31: Effect of Ag NMs on growth of *R. subcapitata* cultures based on protein content at 72 h using probe-sonicated Ag NMs (n=3). Algae were exposed to OECD 201 medium (control), or Ag NMs at concentrations of 20, 50, 80 and 150 µg/L for 72 h. Protein content was then measured using a Bradford assay. Data are expressed as mean protein content ± SD (standard deviation).

3.3.5.2 The growth inhibition of *R. subcapitata* exposed to MWCNTs quantified based on protein content

The protein content in the control samples of *R. subcapitata* was 0.26 (± 0.04) mg/mL. A significant increase in protein content was observed in algae exposed to bath-sonicated MWCNTs at concentrations of 3.5 and 7.15 mg/L for 72 h post exposure. At the highest concentration of bath-sonicated MWCNTs (28.5 mg/L), a significant decrease in protein content was observed compared to the control. The protein content of *R. subcapitata* exposed to 28.5 mg/L of bath-sonicated MWCNTs was 0.159 (± 0.023) mg/mL.

Exposure to all concentrations of probe-sonicated MWCNTs decreased protein content in algae in a concentration-dependent manner. A significant decrease in protein content compared to the control was observed at the two highest concentrations – 3.5 and 7.15 mg/L of probe-sonicated MWCNTs – for which the protein content was 0.13 (± 0.03) and 0.08 (± 0.007) mg/mL, respectively. Overall, probe-sonicated MWCNTs were more toxic to *R. subcapitata* than bath-sonicated MWCNTs when protein content was used as an indicator of toxicity.

Based on the results of two-way ANOVA, the interaction of protein content for both type of NMs (Ag NMs and MWCNTs) were significantly affected by the interaction of the two factors, time points and sonication type (Table 3.4).

Table 3.4: Results of two-way ANOVA interaction results for protein content for both type of NMs (Ag NMs and MWCNTs) for 72h

NMs	Source	DF	Adj MS	F-Value	*P*-Value
Ag NMs	Concentration	6	0.020971	17.11	0.001
Ag NMs	Sonication type	2	0.022969	4.26	0.031
MWCNTs	Concentration	6	0.02379	18.7	0.028
MWCNTs	Sonication type	2	0.01958	3.85	0.027

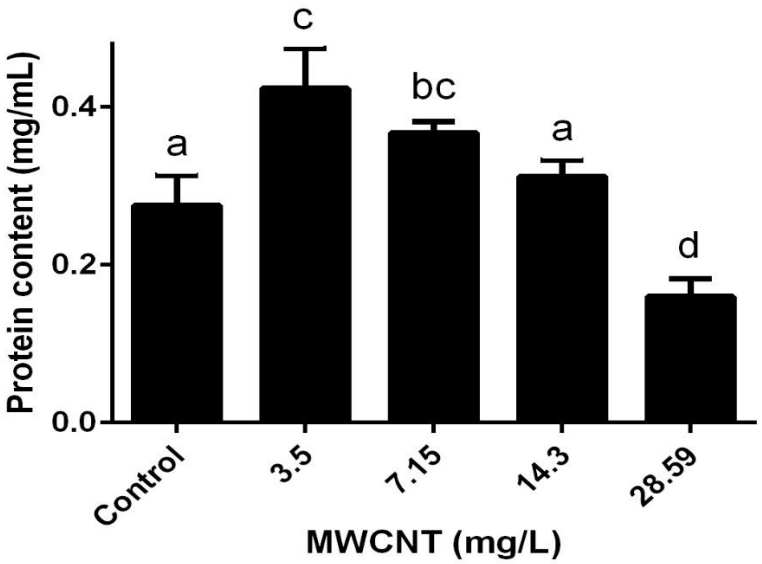


Figure 3.32: Effect of MWCNTs on growth of *R. subcapitata* cultures based on protein content at 72 h using bath-sonicated MWCNTs (n=3). Algae were exposed to OECD 201 medium (control), or MWCNTs at concentrations of 3.5, 7.15, 14.3 and 28.59 mg/L for 72 h. Protein content was then measured using a Bradford assay. Data are expressed as mean protein content ± SD (standard deviation).

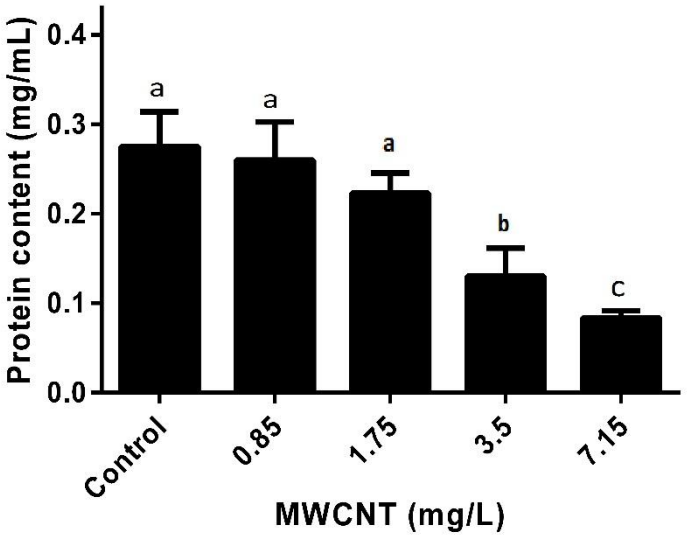


Figure 3.33: Effect of MWCNTs on growth of *R. subcapitata* cultures based on protein content at 72 h using probe-sonicated MWCNTs (n=3). Algae were exposed to OECD 201 medium (control), or MWCNTs at concentrations of 0.85, 1.75, 3.5 and 7.15 mg/L for 72 h. Protein content was then measured using a Bradford assay. Data are expressed as mean protein content ± SD (standard deviation).

3.3.6 *Acute toxicity assessments based on PSII activity*

3.3.6.1 The growth inhibition of *R. subcapitata* exposed to $AgNO_3$ quantified based on PSII activity

After 4 and 24 h of exposure to $AgNO_3$, a significant increase in PSII inhibition was observed in algae across all tested concentrations of bath- and probe-sonicated $AgNO_3$ compared to the control samples. For bath-sonicated $AgNO_3$, the lowest concentration tested (3 µg/L) induced PSII inhibition levels of 62.53% (± 11.59) and 52.35% (± 4.98) after 4 and 24 h of exposure was observed, respectively (Figure 3.34). In addition, the highest concentration tested (25 µg/L) induced a PSII inhibition of 100% and 99.32% (± 1.12) after 4 and 24 h of exposure, respectively.

On the other hand, for probe-sonicated $AgNO_3$, the lowest concentration tested (3 µg/L) induced a PSII inhibition of 52.19% (± 9.58) and 44.65% (± 13.82) after 4 and 24 h of exposure, respectively (Figure 3.34). Moreover, the highest concentration tested (25 µg/L) induced PSII inhibition levels of 94.50% (± 4.36) and 98.92% (± 1.27) after 4 and 24 h of exposure, respectively. Overall, bath-sonicated $AgNO_3$ was more toxic to *R. subcapitata* than probe-sonicated $AgNO_3$ when photosynthesis activity was used as an indicator of toxicity.

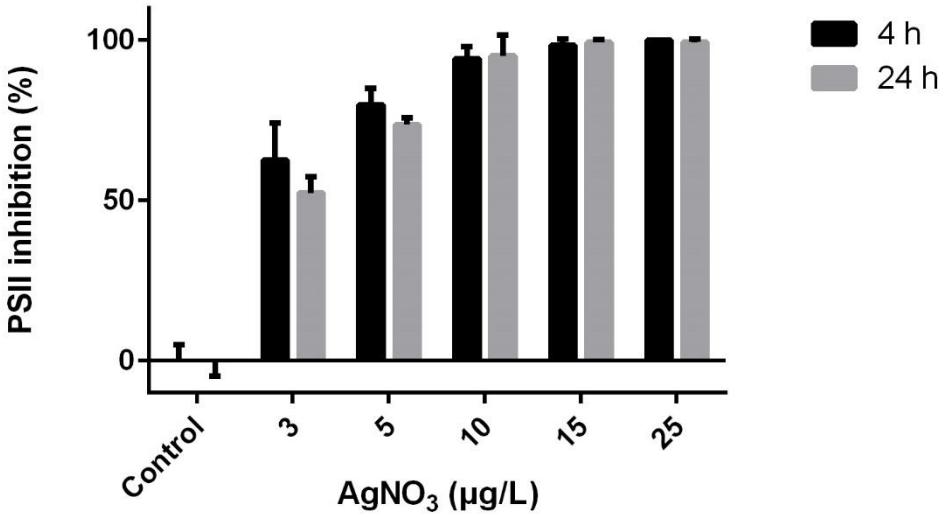


Figure 3.34: Influence of bath-sonicated $AgNO_3$ on the photosynthetic efficiency of *R subcapitata* in OECD 201 medium. Algae were exposed to medium (control), or $AgNO_3$ at concentrations of 3–25 µg/L at 4 and 24 h. Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls); (n=3) and data are mean ± SD (standard deviation).

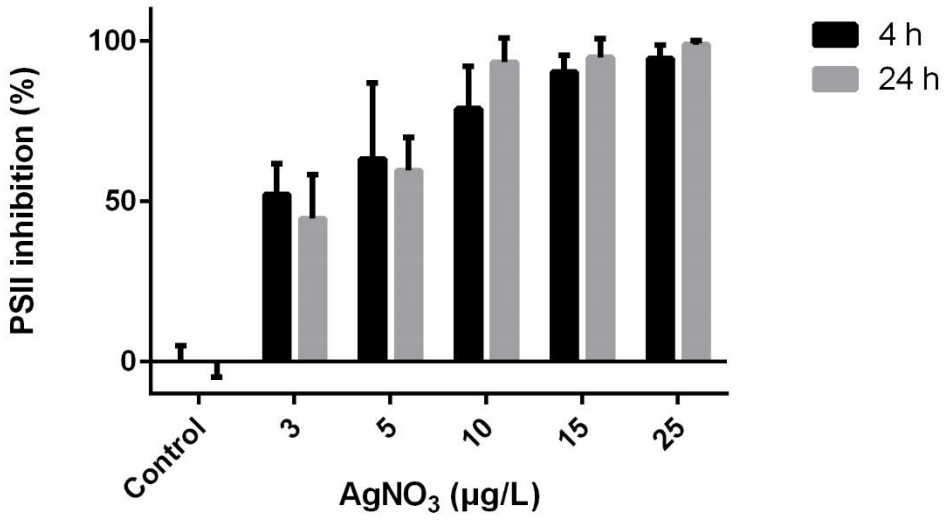


Figure 3.35: Influence of probe-sonicated $AgNO_3$ on the photosynthetic efficiency of *R. subcapitata* in OECD 201 medium. Algae were exposed to medium (control), or $AgNO_3$ at concentrations of 3–25 µg/L at 4 and 24 h. Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls); (n=3) and data are mean ± SD (standard deviation).

3.3.6.2 The growth inhibition of R. subcapitata exposed to Ag NMs quantified based on PSII activity

Growth inhibition of *R. subcapitata* based on photosynthesis activity was observed across all tested concentrations of bath-sonicated Ag NMs except the lowest concentration (20 µg Ag/L) (Figure 3.35). Interestingly, there was slight enhancement of *R. subcapitata* PSII efficiency after 24 h of exposure to 20 µg Ag/L of bath-sonicated Ag NMs. After 4 and 24 h of exposure to bath-sonicated Ag NMs, a significant increase in PSII inhibition was observed at concentrations of 50, 80, 150 and 200 µg Ag/L of Ag NMs compared to the control samples. PSII inhibition at the highest concentration of bath-sonicated Ag NMs was 97.33% (± 1.99) and 95.78% (± 4.31) after 4 and 24 h of exposure to Ag NMs, respectively.

No significant PSII inhibition was observed following exposure of algae to probe-sonicated Ag NMs at concentrations of 20, 50, and 80 µg Ag/L compared to the control samples (Figure 3.36). There was slight enhancement of *R. subcapitata* PSII efficiency after 24 h of exposure to 20 and 50 µg Ag/L of probe-sonicated Ag NMs. However, there was a significant increase in PSII inhibition at concentrations of 150, 200 and 300 µg Ag/L of probe-sonicated Ag NMs, compared to the control (Figure 3.36). After 4 and 24 h of exposure to probe-sonicated Ag NMs, the PSII inhibition levels at the highest concentration (300 µg Ag/L) were 40.51% (± 13.70) and 60.26% (± 13.17), respectively. Overall, bath-sonicated NM300k were more toxic to *R. subcapitata* than probe-sonicated Ag NMs when photosynthesis activity was used as an indicator of toxicity.

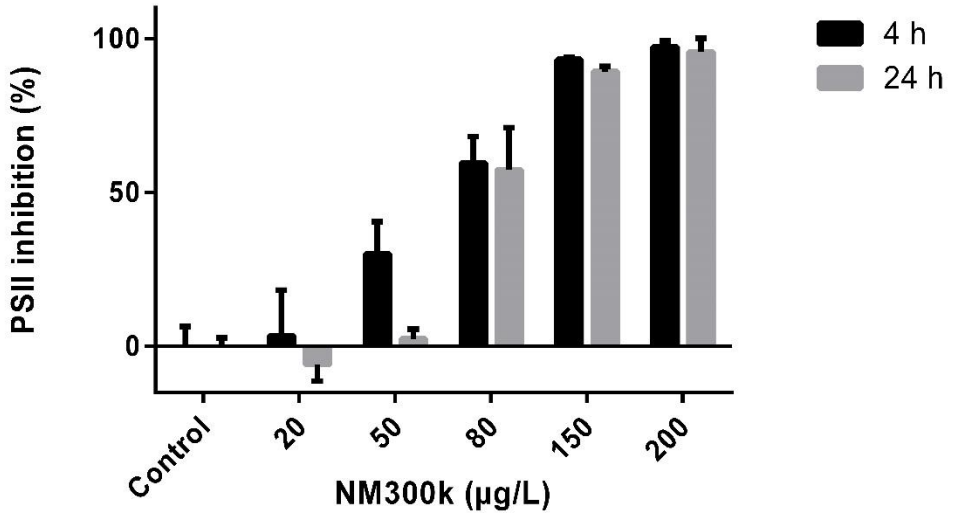


Figure 3.36: Influence of bath-sonicated Ag NMs on the photosynthetic efficiency of *R. subcapitata* in OECD 201 medium. Algae were exposed to medium (control), or Ag NMs at concentrations of 20–200 µg/L at 4 and 24 h. Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls); (n=3) and data are mean ± SD (standard deviation).

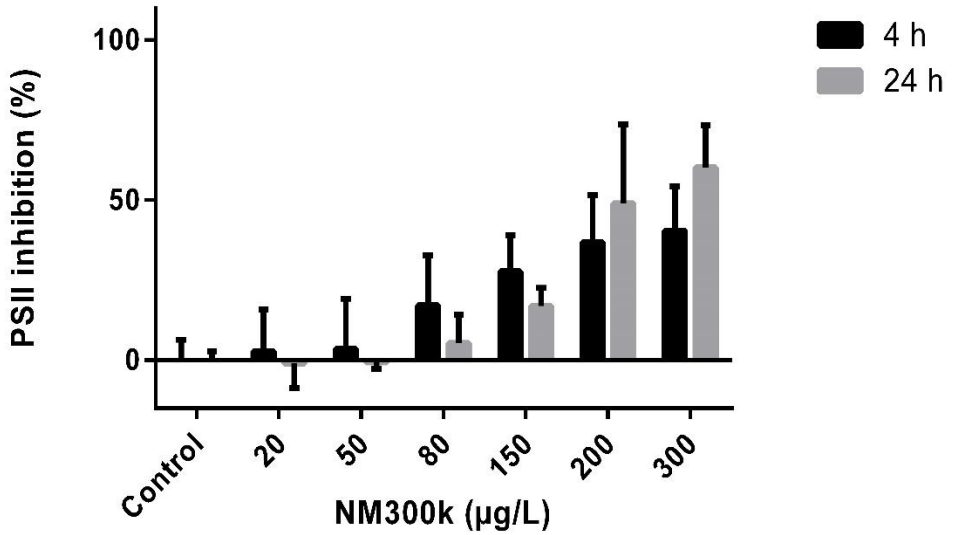


Figure 3.37: Influence of probe-sonicated Ag NMs on the photosynthetic efficiency of *R. subcapitata* in OECD 201 medium. Algae were exposed to medium (control), or Ag NMs at concentrations of 20–300 µg/L at 4 and 24 h. Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls); (n=3) and data are mean ± SD (standard deviation).

3.3.6.3 The growth inhibition of *R. subcapitata* exposed to MWCNTs quantified based on PSII activity

For bath-sonicated MWCNTs, the lowest concentration tested (1 mg/L) induced a slight (but not significant) hormetic effect on the photosynthetic efficiency of *R. subcapitata* at both tested time points, with PSII inhibition levels of 6.12% (± 4.49) and 5.45% (± 4.46) after 4 and 24 h of exposure, respectively (Figure 3.37). At all concentrations of bath-sonicated MWCNTs greater than 1 mg/L, there was a concentration-dependent increase in PSII inhibition. At the highest concentration tested (100 mg/L) of bath-sonicated MWCNTs, the PSII inhibition levels were 77.65% (± 4.81) and 84.40% (± 4.46) after 4 and 24 h of exposure, respectively (Figure 3.38). In terms of probe-sonicated MWCNTs, there was a significant increase in the PSII inhibition of *R. subcapitata* compared to the control samples at all tested concentrations at 4 and 24 h post exposure. At a concentration of 25 mg/L of probe-sonicated MWCNTs, PSII inhibition levels were 41.83% (± 3.06) and 59.45% (± 3.36) after 4 and 24 h, respectively. In addition, at the highest probe-sonicated MWCNT concentration (100 mg/L), PSII inhibition levels were 75.51% (± 11.22) and 84.11% (± 2.21) after 4 and 24 h of exposure, respectively (Figure 3.39).

Overall, probe-sonicated MWCNTs were more toxic to *R. subcapitata* than bath-sonicated MWCNTs when photosynthesis activity was used as an indicator of toxicity. Furthermore, after 4 and 24 h of exposure across all tested suspensions, toxicity can be ranked in decreasing order as follows: $AgNO_3$ > Ag NMs > MWCNTs.

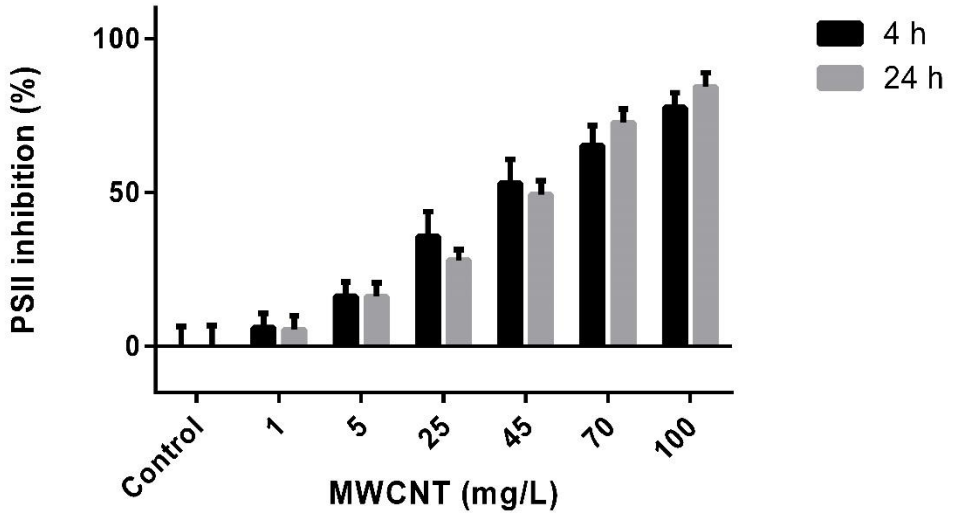


Figure 3.38: Influence of bath-sonicated MWCNTs on the photosynthetic efficiency of *R. subcapitata* in OECD 201 medium. Algae were exposed to medium (control), or MWCNTs at concentrations of 1–100 mg/L at 4 and 24 h. Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls); (n=3) and data are mean ± SD (standard deviation).

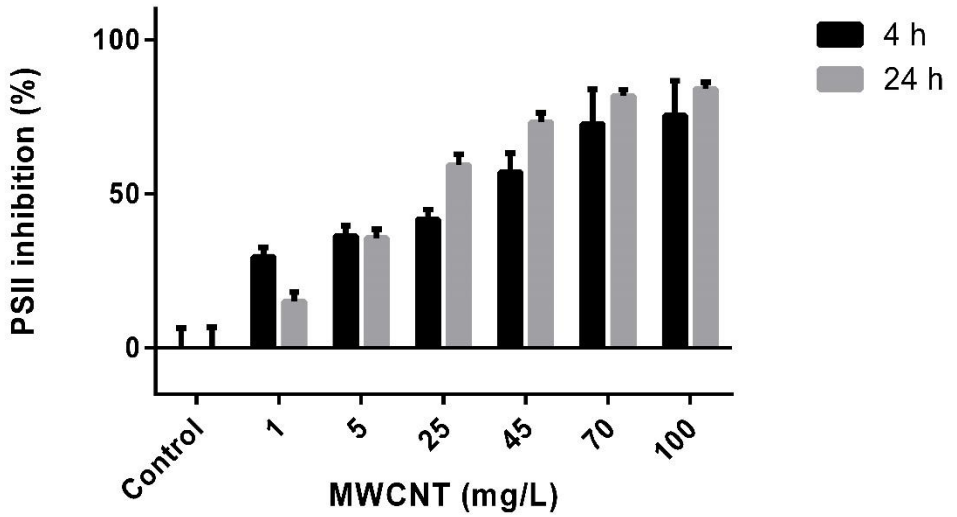


Figure 3.39: Influence of probe-sonicated MWCNTs on the photosynthetic efficiency of *R. subcapitata* in OECD 201 medium. Algae were exposed to medium (control), or MWCNTs at concentrations of 1–100 mg/L at 4 and 24 h. Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls); (n=3) and data are mean ± SD (standard deviation).

Based on the results of two-way ANOVA, the interaction of PSII activity for both type of NMs (Ag NMs and MWCNTs) were significantly affected by the interaction of the two factors, time points and sonication type (Table 3.5).

Table 3.5: Results of two-way ANOVA interaction results for PSII activity for both type of NMs (Ag NMs and MWCNTs) for 72h

NMs	Time	Source	DF	Adj MS	F-Value	*P*-Value
Ag NMs	4 h	Concentration	10	3759.5	28.46	0.018
Ag NMs	24 h	Sonication type	2	7639.2	9.09	0.001
MWCNTs	4 h	Concentration	10	4363.66	46.05	0.042
MWCNTs	24 h	Sonication type	2	5613.87	4.88	0.015

3.3.7 *Interference of tested materials with PSII activity assay*

A test was performed to measure the potential interference of tested materials with photosynthesis activity assay, using a PHYTO-PAM plankton analyser (Figures 3.40–3.45). Photosynthesis measurements were made for all test substances in the absence of algae cells to identify whether they contributed to the photosynthesis readings. Moreover, a control sample with algal cells was run as a positive control (with no tested material) and another sample was used as a negative control (just OECD 201 medium without algal cells).

3.3.7.1 Interference of $AgNO_3$ with PSII activity

The interference of $AgNO_3$ (bath and probe sonicated) with a photosynthesis activity assay is shown in Figures 3.40–3.41. No interference of $AgNO_3$ (both bath- and probe-sonicated suspensions) was observed after 4 and 24 h of exposure across all tested concentrations. However, some background signal coming from culturing medium was detected.

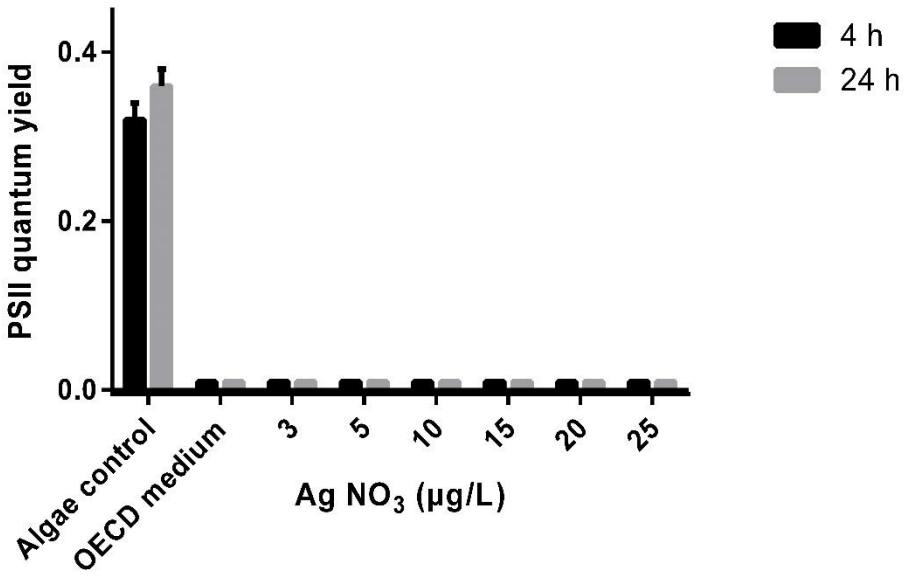


Figure 3.40: Interference of $AgNO_3$ with photosynthetic assay using bath-sonicated $AgNO_3$ (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or $AgNO_3$ at concentrations of 3 to 25 µg/L for 4 and 24 h (without algal cells). Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data are expressed as mean ± SD (standard deviation).

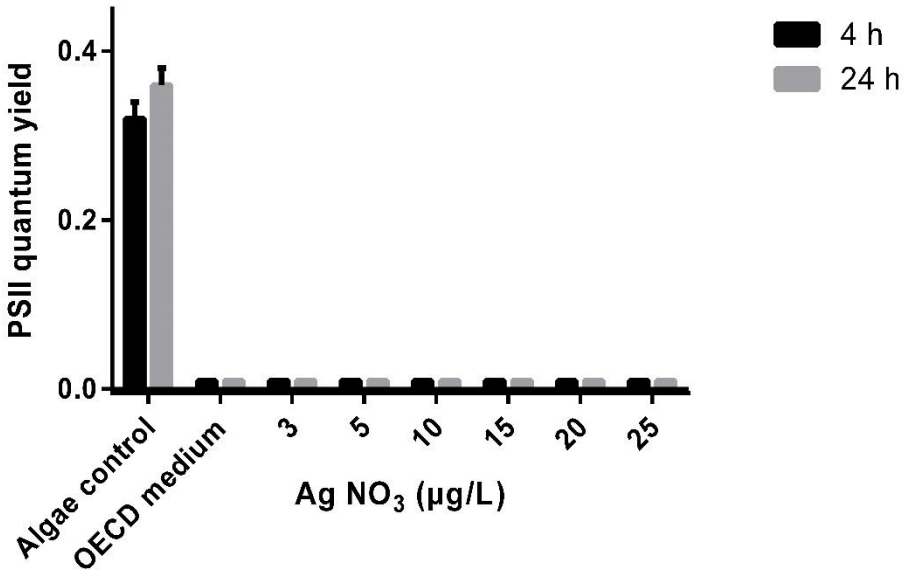


Figure 3.41: Interference of $AgNO_3$ with photosynthetic assay using probe sonicated $AgNO_3$ (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or $AgNO_3$ at concentrations of 3 to 25 µg/L for 4 and 24 h (without algal cells). Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data are expressed as mean ± SD (standard deviation).

3.3.7.2 Interference of Ag NMs with PSII activity

The potential interference of Ag NMs (bath and probe sonicated) with a photosynthesis activity assay is shown in the figures below (Figures 3.42–3.43). No interference of Ag NMs (both bath- and probe-sonicated suspensions) was observed after 4 and 24 h of exposure across all tested concentrations. However, some background signal coming from the culturing medium was detected.

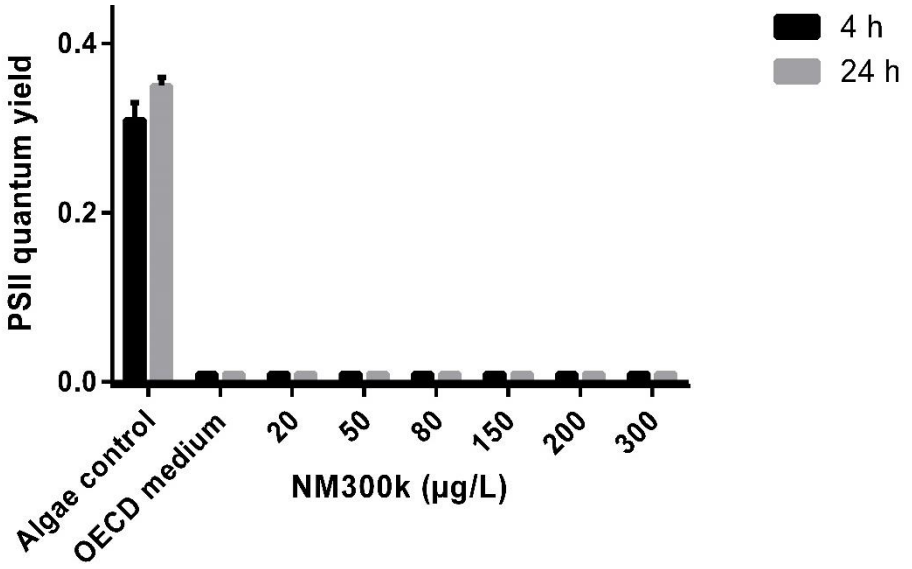


Figure 3.42: Interference of Ag NMs with photosynthetic assay using bath-sonicated Ag NMs (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or Ag NMs at concentrations of 20 to 300 µg/L for 4 and 24 h (without algal cells). Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data are expressed as mean ± SD (standard deviation).

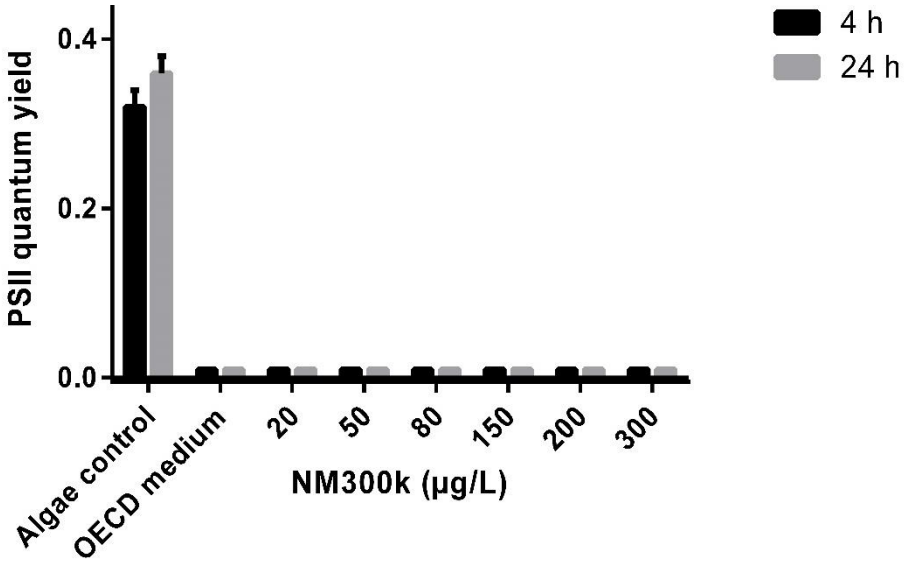


Figure 3.43: Interference of Ag NMs with photosynthetic assay using probe-sonicated Ag NMs (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or Ag NMs at concentrations of 20 to 300 µg/L for 4 and 24 h (without algal cells). Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data are expressed as mean ± SD (standard deviation).

3.3.7.3 Interference of MWCNTs with PSII activity

The potential interference of MWCNTs with photosynthesis activity assay is shown in the figures below (Figures 3.44–3.45). No interference of MWCNTs (both bath- and probe-sonicated suspensions) was observed after 4 and 24 h of exposure across all tested concentrations. However, some background signal coming from the culturing medium was detected.

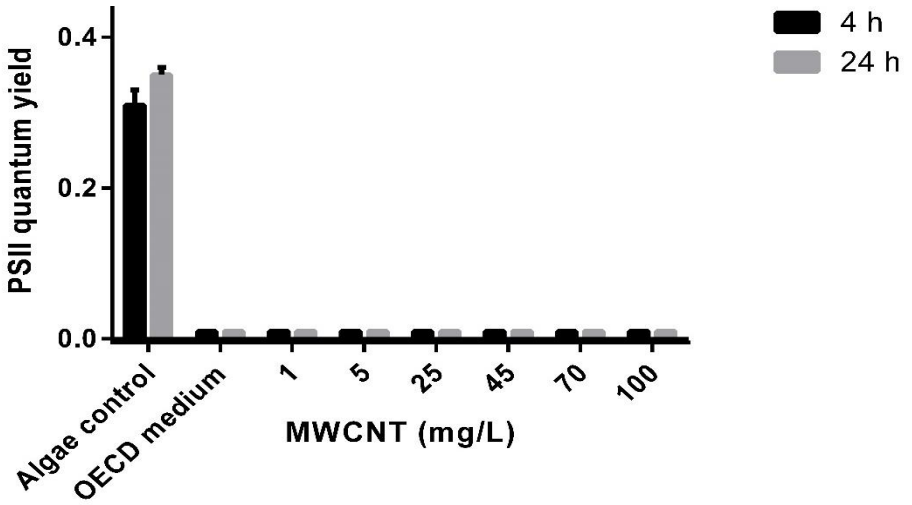


Figure 3.44: Interference of MWCNTs with photosynthetic assay using bath-sonicated MWCNTs (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or MWCNTs at concentrations of 1 to 100 mg/L for 4 and 24 h (without algal cells). Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data are expressed as mean ± SD (standard deviation).

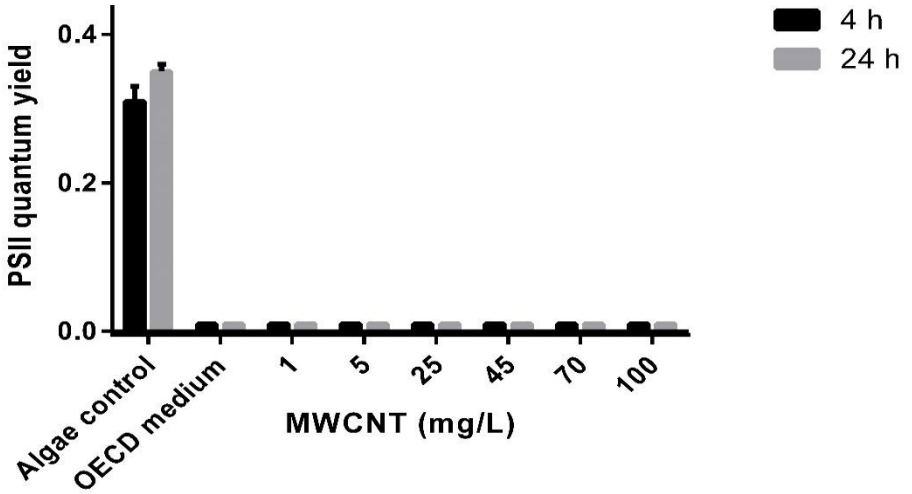


Figure 3.45: Interference of MWCNTs with photosynthetic assay using probe-sonicated MWCNTs (n=3). Treatments were algae exposed to OECD 201 medium (+ control), OECD 201 medium (- control without algal cells) or MWCNTs at concentrations of 1 to 100 mg/L for 4 and 24 h (without algal cells). Photosynthetic efficiency was then measured using a PHYTO-PAM plankton analyser. Data are expressed as mean ± SD (standard deviation).

3.4 Discussion

This study was conducted to evaluate the potential toxicity of different types of NMs – Ag NMs (including dispersant and silver nitrate as a positive control) and MWCNTs – to the freshwater microalga *R. subcapitata* at 3 different time points: 24, 48 and 72 h. Moreover, $AgNO_3$ was used to assess the contribution of ions to Ag NM toxicity. The OECD standard protocol 201 was used to determine algal growth inhibition, which was assessed via measurement of Chl extraction, OD, protein content and photosynthetic activity. This study was also carried out to investigate the impact of the NM sonication dispersion method on NM toxicity to the freshwater microalgae.

Based on Chl a extraction, exposure of *R. subcapitata* to $AgNO_3$ (silver ion control), NM300k and MWCNTs led to an increase in growth inhibition with increasing concentration of the tested materials. However, when exposing this microalga to NM300k DIS (in which the silver NMs were suspended), there were no obvious negative effects. The toxicity ranking based on the findings from all the approaches used in this research study after bath and probe sonication was as follows: $AgNO_3$ > Ag NMs > MWCNTs. Moreover, the Chl extraction assay was one of the most routinely used to assess NM toxicity to algae.

3.4.1 *$AgNO_3$ and Ag NM toxicity*

Both $AgNO_3$ and Ag NMs induced a concentration-dependent inhibition of *R. subcapitata* growth rate, post exposure in OECD medium. The toxicity of silver (ionic and NM) decreased over time, as indicated by higher IC_{50} values at 72 h. As hypothesised, $AgNO_3$ exhibited higher overall toxicity than NM300k. In addition, differences in Ag NMs and $AgNO_3$ toxicity were observed between both types of sonication (bath and probe).

Data generated in this work are comparable to the existing literature on the toxicity of both ionic and Ag NM to algae (see Chapter 1; Table 1.1). $AgNO_3$ has an IC_{50} value in algae in the range of 9.9–31.9 µg/L, depending on experimental conditions (e.g. species, exposure time, medium composition, and endpoint). As observed in the work presented in this chapter, Ag NMs have a higher algal IC_{50} value relative to $AgNO_3$, in the range of 20–1,600 µg/L (see Chapter 1; Table 1.1). This observed IC_{50} range for Ag NMs is also affected by the same experimental conditions as for $AgNO_3$, in addition to dispersion method and NM characteristics.

The reported variation in toxicity for Ag NMs is higher than that reported for $AgNO_3$. Such differences are likely to derive from the use of Ag NMs of varied physicochemical properties (such as coating, size, shape) in existing studies, the use of different sonication methods to prepare and disperse NMs, the influence of agglomeration and aggregation and the methodology used to assess toxicity (such as medium selection, time point investigated, species tested and cell density).

It can be observed that $AgNO_3$ dispersed using probe sonication was approximately 3 times more toxic than the Ag NM suspension prepared with bath sonication after 24 h of exposure, and the difference between the results (IC_{50}) decreased over the exposure time. A similar toxic potency of $AgNO_3$ has been determined by other researchers. Navarro et al. (2008b) found the IC_{50} values for $AgNO_3$ to *Chlamydomonas reinhardtii* were 31.9 and 31.2 µg/L after exposure for 1 and 2 h, respectively. In the same study, the authors investigated the impact of carbonate-coated Ag NMs on growth inhibition of the same microalga, and concluded that the IC_{50} values were 356 and 89 µg/L after 1 and 5 h of exposure. This study looked at limited time points, and therefore consideration of toxicity over longer periods would be recommended to make comparisons between the toxicity of $AgNO_3$ and Ag NMs.

Using the same standard experimental design (OECD 201 test, OECD medium, Chl extraction) with *Chlorella vulgaris*, Kalman et al. (2015) found a mean 72-h IC_{50} value of 9.3, 9.2 and 49.3 µg/L Ag for Ag NMs coated with polyvinylpyrrolidone (PVP), citrate and polyethylene glycol (PEG), respectively. In the same study, the 72-h IC_{50} value for $AgNO_3$ was 5.3 (± 0.5) µg/L Ag, indicating that *C. vulgaris* shows a similar sensitivity to $AgNO_3$ to that shown by *R. subcapitata*.

Griffitt et al. (2008) found that the IC_{50} value for Ag NM, size 20–30 nm coated with thin layers (~2–5 nm) of metal oxide, was 190 µg/L. It is possible that their result was slightly different from this research because their NMs were coated, whereas in this study the Ag NMs was uncoated. Furthermore, the medium and suspension method used are likely to have an impact on this difference. Research by Navarro et al. (2008a) on the effects of carbonate-coated Ag NMs on algal growth and photosynthesis found an IC_{50} value of 1.037 mg/L at 1 h and 329 mg/L at 2 h for growth inhibition of Ag NM. However, they used *Chlamydomonas reinhardtii* as a test species and incubated in a water bath at room temperature, as well as different time points and medium.

Ribeiro et al. (2014) found a lower toxicity of $AgNO_3$ to *R. subcapitata* (72-h IC_{50} = 33.79 µg/L Ag), however a difference in experimental design may have affected toxicity estimates (e.g. initial cell density or test vessels). Few studies have specifically assessed the toxicity of NM300k to *R. subcapitata* (Wang et al. 2012; Sorensen and Baun, 2014). Sorensen and Baun (2014) found that in OECD 201 medium, $AgNO_3$ and NM300k caused a 48-h growth inhibition (IC_{50}) of 4.9 and 140 µg Ag/L, respectively. In the work presented in this chapter, at comparable time points, the 48-h IC_{50} values were 10.06 and 8.79 µg Ag/L for bath- and probe-sonicated $AgNO_3$, respectively, and 44.09 and 95.51 µg Ag/L for bath- and probe-sonicated NM300k, respectively, indicating higher toxicity. However the test design in Sorensen and Baun's (2014) work was in a miniaturised version of the standard OECD 201 test (Arensberg et al. 1995), which may have accounted for the lower sensitivity observed. Furthermore, it is worth noting that from a risk-assessment perspective, this difference in toxicity observed between studies is not particularly large.

Regarding the effect of NM300k DIS on algal growth, no significant negative effect was determined, which indicates that this is not toxic to *R. subcapitata* at the equivalent concentrations tested.

3.4.2 *MWCNT toxicity*

In terms of the toxicity of MWCNTs for *R. subcapitata* growth based on Chl a extraction, the IC_{50} values determined were 28.59 mg/L and 6.83 mg/L for bath and probe sonication, respectively. Using probe sonication led to higher dispersibility of the MWCNTs than using bath sonication. Comparing these findings with previous studies, they seem to be similar to the results of Schwab et al. (2011).

In the agglomerated suspension of MWCNTs, Schwab et al.'s (2011) results indicate that the determined IC_{50} values for *C. vulgaris* and *R. subcapitata* were 24 and 36 mg/L, respectively; these values are close to the results obtained in this study for the bath-sonicated sample. Moreover, Schwab et al.'s findings indicate that the IC_{50} values to *C. vulgaris* and *R. subcapitata* were 1.8 and 20 mg/L, respectively in well-dispersed MWCNTs using NOM. In this study, the obtained IC_{50} value using probe sonication without using NOM was 6.83 ± 0.34 mg/L; this value is close to the finding of Schwab et al. (2011). The small difference between these values could be due to the bath-sonication time; Schwab et al. sonicated the MWCNTs for 2 × 15 min, and in this study just 2 × 8 min were used. In terms of well-dispersed MWCNTs, Schwab and

colleagues sonicated the MWCNTs for 2 × 15 min, then further ultrasonicated for 6 × a total of 15 min, and in this study just 14.11 min, probe-sonicated MWCNTs were used.

Exposure of *Chlorella sp*. as a model organism to MWCNTs for 96 h resulted in similar IC_{50} values to a study conducted by Long et al. (2012). These authors used 3 different outer-diameter size ranges of MWCNTs (<10, 20–40 and 60–100 nm) and the IC_{50} values were 41, 12.7 and 12.4 mg/L, respectively. One reason for this difference compared with the results obtained in this study could be the exposure time point (72 vs 96 h). The other possibility for this difference might be the use of different types of algae species – *R. subcapitata* was potentially less sensitive to CNTs than *C. vulgaris* (Schwab et al. 2011) – or, of course, the use of MWCNTs with different physicochemical properties. In general, MWCNTs were more toxic when probe sonicated than when they were bath sonicated.

Furthermore, the addition of locust gum was helpful to precipitate out the NMs, as well as the results of cell destructions in order to avoid potential NM interference with fluorescence measurements, and the extraction in acetone only in order to simplify the extraction procedure.

3.4.3 *Bath vs probe sonication*

In this study, probe sonication led to an increase in the toxicity of MWCNTs and a decrease in the toxicity of Ag NMs based on Chl a extraction. Bath-sonicated MWCNT suspensions inhibited growth of *R. subcapitata* significantly more than probe-sonicated MWCNT suspensions based on OD, whereas the opposite effect was observed for ionic control ($AgNO_3$). Furthermore, the findings from this study suggested that the bath-sonicated NMs are less well dispersed (agglomerated suspension of NMs), which led to less toxicity, and the probe-sonicated NMs are well dispersed, which led to greater toxicity.

An increase in toxicity after probe sonication of MWCNTs may be related to changes in their length (Huang et al. 2002). To test this hypothesis, TEM analyses were performed on samples after two types of sonication process for NMs (see Chapter 2). It was found that there were NMs agglomerated after bath sonication. However, well-dispersed NMs were observed after probe sonication (in the case of both Ag NMs and MWCNTs).

Ag NMs is highly toxic to *R. subcapitata*, with bath sonication resulting in higher toxicity than probe sonication. However, the obtained results from chapter 2 confirm that the probe sonication is more effective on NMs agglomeration rather than bath sonication, leading to ease of the interaction of individual NM with algal cell. This might be explained by the fact that Ag NMs could have become stuck on vial walls during the probe sonication, somehow resulting in lower availability. Another possible reason for this lower toxicity is that Ag NMs interacted with the probe sonicator tip, which may lead to loss of some amount of Ag NMs in the stock suspension. In addition, Ag NMs could be lost to the surface of the storage container. Furthermore, when using higher amplitudes, probe sonication can be destructive to the solid NMs (Hielscher 2005).

3.4.4 *OD*

The effect of $AgNO_3$ and Ag NMs on growth inhibition of *R. subcapitata* was assessed by measuring the OD of algae cultured in flasks. The findings indicate a good reproducibility and indicated a high toxicity for $AgNO_3$ and Ag NMs. Comparing IC_{50} values obtained from OD, with the growth rate obtained by Chl extraction, quantified this effect and allowed a direct surrogate to be obtained for biomass density. The findings in this study were lower than the findings from a study conducted by Książyk et al. (2015). They assessed the inhibition of algal growth after 72 h of exposure relative to controls by measuring OD at 670 nm. The commercial Algaltoxkit FTM (Creasel, Belgium) was used, and the initial density of cells was 10^6/mL. Their results confirm that at ≥5 mg/L, Ag NM concentrations were found to cause a 100% drop in algal cells. Their study seemed to indicate lower toxicity of the Ag NM tested. However they used a different method (commercial Algaltoxkit FTM). In addition, the authors indicate that exposure media were sonicated for 30 min before the toxicity tests.

In terms of MWCNTs, the IC_{50} values were 40.28 and 103.76 mg/L for bath- and probe-sonicated MWCNTs, respectively. These results obtained from OD experiments are double of the IC_{50} value obtained from Chl extraction experiment for bath-sonicated MWCNTs and 15 times higher than the IC_{50} value obtained for probe-sonicated MWCNTs.

However, with a high concentration of NMs, there was interference by the NMs in the OD readings. This finding comes in parallel with a study conducted by Rodea-

Palomares et al. (2011). They investigated the ecotoxicological effects of CeO_2 NMs on *R. subcapitata*; the growth inhibition of *R. subcapitata* was assessed by measuring the OD of algae cultured in a microplate, in parallel with cell counting. The observed effect on the growth rate of *R. subcapitata* when using OD was particularly high. Furthermore, the IC_{50} values were low because of the formation of algal cell agglomerates with CeO_2 NMs.

3.4.5 *Protein content*

The lowest concentration of probe-sonicated NM300k (20 µg/L) and bath-sonicated MWCNTs (3.5 mg/L) significantly increased the protein content, compared to the control samples. This might suggest that the probe-sonicated NM300k at low concentrations stimulates the growth of *R. subcapitata*. Jiang et al. (2014) investigated the effect of Ag NMs on the protein content of higher plants (*Spirodela polyrhiza*) using a Bradford assay. Their findings confirm that the protein content increased with low Ag concentration and then decreased with higher Ag concentration (10 mg/L), which is in keeping with the findings of this research study. Moreover, the findings of this study confirm that the highest tested concentration of Ag NMs decreased the protein content, indicating that a high concentration of Ag NMs damaged protein generation or accelerated protein decomposition, likely because of oxidation (Jiang et al. 2014).

3.4.6 *PSII activity*

Short-term (<24-h) PSII efficiency inhibition has previously been demonstrated to be in good agreement with 72-h growth inhibition in a range of chemical toxicants (Masojídek et al. 2011). IC_{50} data have also been successfully generated from other PSII test systems at time points <24 h, but not compared to growth inhibition (Snel et al. 1998; Juneau et al. 2003). Furthermore, short-term testing (<24 h) may mitigate potential variability in exposure concentrations used in tests of system-toxicant interactions in longer-term (>24-h) Ag NM exposures (Sorensen and Baun 2014). Assessment of PSII efficiency at 4 and 24 h proved a suitable endpoint for comparisons with 72-h growth inhibition tests for Ag, although sensitivity was lower in PSII tests (Curry 2017).

Wang et al. (2012) found a 4.5 h PSII inhibition IC_{50} value of 928.74 µg Ag/L for Ag NMs; however PSII inhibition tends to be a less sensitive endpoint than growth

inhibition (Navarro et al. 2008a). Assessment of PSII efficiency evaluates productivity, whereas growth inhibition assesses biomass. Thus, assessment of NM toxicity to algae is influenced by the selection of the endpoint used to assess algal growth, and can make it challenging to compare findings from different studies. Where possible, it is recommended that studies should aim to include a standard algal growth inhibition test such as the OECD 201 test in nanotoxicology studies, using Chl measurements. However the lack of standard protocol for PSII toxicity tests, coupled with the wide range of potential parameters that can be measured, drive the need for harmonisation of approaches (Ralph et al. 2007). It is therefore recommended that assessment of PSII is investigated more widely when investigating the impacts of chemical substances on algae.

The findings of this study are in correspondence with those of Navarro et al. (2015), who investigated the effects of $AgNO_3$ and different coated Ag NMs on the PSII of *C. reinhardtii*. Their results confirm that in all cases the excess cysteine completely prevented the effects of Ag NMs on photosynthetic yield compared to $AgNO_3$. This finding can confirm the role of Ag ions as a cause of the observed effect on photosynthesis in their study.

3.4.7 *Limitations of assays*

Although the OD study has successfully demonstrated the effects of $AgNO_3$ on microalgal *R. subcapitata*, it has certain limitations in terms of interference with NMs. Regarding patterns in the toxicity of $AgNO_3$ on *R. subcapitata* based on Chl a extraction, the IC_{50} values were 14.09 and 11.90 µg/L for bath- and probe-sonicated $AgNO_3$, respectively; the IC_{50} values of $AgNO_3$ based on OD were 14.78 and 12.05 µg/L for bath- and probe-sonicated $AgNO_3$, respectively. However, in the presence of NMs, the toxicity patterns of NMs on *R. subcapitata* gave different results, particularly with MWCNTs. The IC_{50} values, based on Chl a extraction, were 28.59 and 6.83 mg/L for bath- and probe-sonicated MWCNTs, respectively; and the IC_{50} values of MWCNTs based on OD were 40.28 and 103.76 mg/L for bath- and probe-sonicated MWCNTs, respectively. The principal limitation of this assay was the interference of NMs with OD measurements, specifically with the highest concentration of Ag NMs (300 µg/L) and across all tested concentrations of MWCNTs (mg/L). Generally, NMs interfered with OD measurements, with MWCNTs doing so to a greater extent, given their larger concentration in this study.

Some studies have justified the use of acid-washed glassware in order to prevent carry-over of compounds from previous uses interfering with silver speciation (Wang et al. 2012; Navarro et al. 2015). Sekine et al. (2015) found that not only does glass sorb more free silver (Ag^+) than plastic (polypropylene and polycarbonate), but this effect can be magnified in glass flasks with a larger surface-area-to-volume ratio. Furthermore, Ag NM adsorption is highly dependent on the physicochemical properties of the NMs themselves, and the composition and size of the test vessel.

In terms of shared equipment, it is important to wash and clean thoroughly any shared equipment such as probe sonicators before and after use, to avoid cross-contamination. In the case of probe sonication there are a few important factors that may affect the container in which the NMs are suspended (Booth and Jansen, 2015). The crucial elements are the shape, size and material of the container. The probe sonicator tip cannot touch the walls and bottom of the containers. The sonication container could move and contact the probe tip during the sonication process (Booth and Jansen, 2015). It is important to use a clamp to stabilize the vial during the sonication process to avoid contact of the probe tip with the container.

3.5 Conclusion

Most studies investigating Ag NMs and algae also investigated the toxicity of $AgNO_3$ in their test systems (Navarro et al. 2008b; Wang et al. 2012; Ribeiro et al. 2014; Kalman et al. 2015; Sorensen and Baun, 2014). The inclusion of ionic controls in NM toxicity studies of this nature should be considered as best practice; due to the highly variable properties of NMs and potential artefacts associated with the preparation method, soluble ionic controls represent a more comparable benchmark toxicant for the comparison between both tests and particles. In addition, data generated by the use of ionic controls may begin to address hypotheses regarding the relative contribution of dissolved ions in NM toxicity studies.

One of the strengths of this study is that it represents a comprehensive examination of the effects of some tested materials on algae using 4 different methods: Chl a extraction, OD, protein content and PSII efficiency. The method used does potentially affect the outcome of the hazard test. Chl extraction is a rapid method of quantifying growth in algal studies, which has been demonstrated to be more sensitive than absorbance in OD measurements (Geis et al. 2000; Van Wagenen et al. 2014). It was observed, however, that Ag NMs and MWCNTs do not interfere with the method used

for total chlorophyll extraction. However, NMs can interfere with the method used for OD measurements. Based on the findings, it is recommended to use a Chl extraction assay in the future to assess NMs' toxicity to algae.

Published work to date has investigated the effects of silver NMs and CNTs on freshwater microalgae, using stirring or bath/probe sonication, with some studies adding dispersants such as NOM (Griffitt et al. 2008; Schwab et al. 2011). Furthermore, no studies have compared the toxicity of Ag NM or MWCNTs with bath and probe sonication dispersion protocols. It is of note that previous studies were carried out using stirring or bath sonication to disperse the NMs prior to exposure. Furthermore, most existing studies have focussed on test concentrations in the mg/L range (Kermanizadeh et al. 2012; Wang et al. 2012; Hougaard et al. 2013; Kermanizadeh et al. 2013; Völker et al. 2013; Sorensen and Baun 2014; Sauer et al. 2015; Kleiven et al. 2018). However, little data exists on NM properties in the µg/L range, which are hazardous at low concentrations.

Chapter 4

Interaction and uptake of NMs with algae

Chapter 4 **Interaction and uptake of NMs with algae**

4.1 Introduction

4.1.1 *The microalgae R. subcapitata*

Among aquatic organisms, the unicellular microalgae are important in nano-ecotoxicity studies because they are primary producers and represent the base of aquatic food webs (Sournia 1978). To evaluate the NM effects on the aquatic environment, it is necessary to determine the interaction, uptake and accumulation of NMs in algae.

Microalgae are single-cell organisms surrounded by an inner plasma membrane and an outer cell wall of various compositions (Yue et al. 2017). Algal cell walls are remarkably diverse among different species in their biochemical compositions and structural features. Some algae have cell walls that are similar to typical terrestrial plant cell walls (Sorensen et al. 2010; Sørensen et al. 2011) which are comprised of networks of cellulose microfibrils and cross-linking glycans. Other algae, for instance the alga *Chlamydomonas reinhardtii*, have less cellulose but mainly glycoproteins in their cell walls which are composed of multiple crystalline layers of about 100 nm in thickness (Monk et al. 1983; Nakano et al. 1987; Leander et al. 2001; Leander and Farmer 2001). The algal cell wall composition and structure can undergo dynamic changes during the different stages of cell development (Yamagishi et al. 2017).

The pores in such cell walls have a size estimated to be 5–20 nm. These pores can help the algae to maintain integrity, and constitute a primary site for interaction with the surrounding environment (Fleischer et al. 1999).

4.1.2 *Interaction of NMs with algae*

For NMs to enter algal cells, they must pass first through the cell wall and subsequently through the plasma membrane via endocytotic processes or passive diffusion. The algal cell wall is semi permeable; however, little is known about the pore size. Large NMs that are above the size of the pores might be excluded from passing through the cell wall (Navarro et al. 2008a). The diversity in algal cell wall composition and structure may influence the passage of the NMs into, and through, the cell wall. Few studies have demonstrated endocytosis in algae. The permeability of the cells can change during their life cycles. As shown in the alga *H. pluvialis*, some

particular molecules were found to be taken up by cells exclusively during cell division (Praveenkumar et al. 2015).

The cell wall may have an increased porosity during the growth period, due to the insertion of newly synthesised wall materials (De Nobel and Barnett 1991; Yamamoto et al. 2003). Moreover, the adsorption of NMs to the cell surface, or dissolved metal ions, might cause damage to the cell walls or membranes (Machado and Soares 2015). It is not known yet whether the changes in cell permeability will facilitate NM internalisation.

As shown in a systematic study with the alga *C. reinhardtii* wild type, neither Ag NM nor cerium dioxide NMs were evidenced to be internalised by the algal cells, as measured by inductively coupled plasma mass spectrometry (ICP-MS) (Piccapietra et al. 2012; Röhder 2014). This suggests that both the cell wall and the cell membrane may hinder the NM entry. Using hyperspectral imaging, particulate forms of Ag were found to be intracellular in Ag NM-exposed *C. reinhardtii* cells, yet the presence of NMs was attributed to the reduction or precipitation of Ag^+ that was released from Ag NM, rather than a direct uptake of Ag NM in the exposure medium (Barwal et al. 2011; Leclerc and Wilkinson 2013). Some studies reported that NMs were gathered onto the algal cell wall (Hoecke et al. 2008; Hoecke et al. 2009; Hartmann et al. 2013), however, it is not clear whether the NMs have direct contact with the cells.

4.1.3 *Cell wall as barrier in microorganisms*

The cell wall can be envisioned to be an efficient barrier to combat the penetration of NMs into cell-wall-bearing cells (Lead et al. 2018). However, there are few mechanistic studies that attempt to understand the extent to which cell walls provide a protective function, or which structures and functions are protective. Schwab et al. (2016) listed some important factors strongly affecting NMs' internalisation into phytoplankton and algal cells, such as cell wall composition, mucilage, symbiotic microorganisms (mycorrhiza), the absence of a cuticle (aquatic plants) and stomata apertures (pores).

More often, NM uptake was not evidenced in algae (Hoecke et al. 2009; Piccapietra et al. 2012; Leclerc and Wilkinson 2013; Röhder 2014), which emphasises the role of the algal surface as a barrier against NM entry to the cells.

Accordingly, internalisation of NMs in algae was suggested in only a few studies (Wang et al. 2013; Taylor et al. 2015). In contrast, there was no evidence of NM uptake

into algae in many other studies using electron microscope imaging and/or analysis of internalised metal in cells (Hoecke et al. 2009; Piccapietra et al. 2012; Behra et al. 2013; Leclerc and Wilkinson 2013; Röhder et al. 2014). Moreover, the comparatively thick cell wall of algae appears to be a significant barrier to NM uptake (Lead et al. 2018).

In a study of the mechanism of chronic toxicity of CeO_2 NMs to the microalga *R. subcapitata*, Angel et al. (2015) used hyperspectral imaging to demonstrate the coating of cells with NMs. Coating cells in this way could cause cell damage (Rogers et al. 2010). Coating was absent in the presence of dissolved organic carbon (humic acid), minimising toxicity. Even though CeO_2 and TiO_2 are sources of ROS, the intensity of ROS production was found to be little, and not sufficient to cause oxidative damage (Angel et al. 2013).

4.1.4 *Interactions of NMs with microalga R. subcapitata*

The high surface-to-volume ratio of NMs greatly favours the adsorption of proteins present in surrounding fluid. Proteins have different functional groups, for example: hydroxyl; carboxylate; amine; sulfhydryl, and phosphate, which offer a range of active sites to interact and bind with NMs (Lynch et al. 2007; Lynch and Dawson 2008; Monopoli et al. 2012). In contrast, adsorption of proteins to the NM surface may affect the NM's stability. The surface charge of NMs might be either neutralised, if adsorbed proteins possess the opposite electrical property, or enhanced, if the protein is carrying the same charge. Changes of surface charge will further affect the stability of NMs (Yoo et al. 2008) (more details in Chapter 2). Furthermore, the concentration of proteins was found to affect the stability of NMs, with more agglomerates formed in the presence of a higher concentration of proteins (Meißner et al. 2010).

Some researchers indicated some factors to understand a metal–organism interaction in aquatic environments (Tessier et al. 1993; Campbell 1995; Lee et al. 2004). These were: firstly, metal availability in the environment; secondly, the route of metal uptake by the organism, and thirdly, the biological effects of the metal on the organism.

The biotic ligand model (BLM), a common model for metal–organism interactions, assumes that the free-ion concentration of a metal is the primary factor determining dissolved metal uptake and toxicity and, thus, predicts that the bioavailability of Ag will be positively related to the concentration of the free ion among various Ag complexes in medium (Morel 1983).

Focusing on the interaction of Ag NMs with unicellular algae, Li et al. (2015b) and Piccapietra et al. (2011) confirmed a strong interaction of materials with the cell surface of *Chlamydomonas reinhardtii* and *Euglena gracilis*, respectively, but with negligible uptake.

4.1.5 *Uptake and accumulation of metal-based NMs*

Internalisation of NMs in algae was suggested in only a few studies (Miao et al. 2010; Wang et al. 2013; Jiang et al. 2014; Kalman et al. 2015; Li et al. 2015b; Taylor et al. 2015; Zhou et al. 2016; Xia et al. 2018), all of which used metal-based NMs that tend to release metal ions. For instance, Ag NMs were visualised inside the cell-wall deficient alga *O. danica* (Miao et al. 2010), *Chlorella vulgaris* (Kalman et al. 2015), *Euglena gracilis* (Li et al. 2015b) and *Chlorella pyrenoidosa* (Zhou et al. 2016) using TEM imaging.

Fleischer et al. (1999) investigated the role of pore size for crossing some types of carbohydrates on algal cell walls (*Chenopodium album*) and confirmed that algal cell walls have pores of a few nm diameter, between 5 and 20 nm. Moore (2006) suggested that only NMs that are smaller than the size of the pores (<20 nm) may be internalised crossing the cell wall.

In a recent study, Ribeiro et al. (2015) investigated silver uptake by *R. subcapitata* in its NM form or in the dissolved or ionic form (Ag^+) using Coherent anti-Stokes Raman Scattering (CARS) microscopy as a powerful optical microscopy technique with several advantages over conventional biological imaging techniques: label-free contrast, increased depth penetration and reduced phototoxicity (Rodriguez et al. 2006; Evans and Xie 2008). In this study algae were previously exposed to both Ag NM and $AgNO_3$ at 15 mg Ag/L and sampled accordingly. The treatments consisted of: (1) suspended algae were sampled and centrifuged at 2,862 g for 3 min, then washed 3 times with Milli-Q water and fixed in a single-strength glutaraldehyde fixative (4%) in cacodylate buffer at room temperature for 4 h; (2) algae were sampled then centrifuged at 2,862 g for 3 min (without pellet washing) and proceeded to fixation; (3) suspended algae cells were placed straight in the fixative (without centrifugation or washing). After the fixation period, all different algae treatments were placed on a microscope slide with a cover slip. *R. subcapitata* exposed to Ag NMs showed no evidence of internalisation of the NMs into the algal cells used in this experimental setup. Agglomerates varying in size could be visualised externally,

with the size of the agglomerate being reduced in response to the washing process. Additionally, CARS images show a lack of association of Ag NMs with the algae. All Ag NM signals were shown to be from outside the cell after 3D image sectioning with CARS, confirming that no particles were uptaken by the algae. The authors suggest that internalised Ag was in the form of ionic or dissolved Ag.

As an example of inorganic granular biopersistent NMs, CeO_2 NMs were shown to adsorb to phytoplankton, an important food source for marine organisms, within 1 h of exposure at 0, 1, 2 and 3 mg/L. Electrostatic attraction between the charged surfaces was assumed as a mechanism rather than active uptake or a chemical process (Conway et al. 2014).

In a study performed with the freshwater algae *R. subcapitata*, up to 38% of the total cerium became directly associated with the cells during the 72 h exposure to concentrations between 15 and 200 μg/L of polyacrylic-acid-stabilised CeO_2. At the same time, the concentration of dissolved cerium in the exposure suspensions was very low (0.5–5.6 μg/L), suggesting that toxicological effects derived from dissolved ions are minimal (Booth et al. 2015). Both results confirm an affinity of CeO_2 for algal cells; however, this may depend on exposure concentration and particle modification.

The freshwater microcrustacean, *Daphnia pulex*, was shown to accumulate, in a water suspension of 10 mg/L of CeO_2 NMs, to a total amount of 24 ± 5% Ce/g dry weight when fed with algae (*Chlorella pseudomonas*) (Auffan et al. 2013). In contrast, a reduced uptake of 7 ± 3% Ce uptake/g dry weight was recorded when no algae were given. This demonstrates the importance of CeO_2–algae interactions for NM uptake by daphnids. It was further demonstrated by X-ray fluorescence and SEM that the shedding of the chitinous exoskeleton is the main mechanism governing the release of CeO_2 by *D. pulex* independent of the feeding regimen. This confirms the crucial role of chitin for transfer of NMs in aquatic environments (Auffan et al. 2013). There have also been reports of a relationship between metal ion uptake and NM biokinetics in *D. magna* (Tan and Wang 2014; Tan et al. 2016). In Table 4.1, there is a summary of selected examples showing adsorption/interaction and internalisation of metal-based NMs by microalgae.

Table 4.1: Selected examples showing adsorption/interaction and internalisation of metal-based NMs by microalgae

No.	NMs	Size (nm)	Medium	Microorganism	Additional information	Reference
1	Ag NMs	50	Blue-Green Medium (BG11) and Seawater	*Chlorella vulgaris* (freshwater) *and Dunaliella tertiolecta* (marine)	Morphological changes in *Chlorella vulgaris* and *Dunaliella tertiolecta* compared to control. More cell aggregate formation compared to control cell in both algae	(Oukarroum et al. 2012a)
2	Zn NMs	Needle length <100	Blue-Green Medium (BG11)	*Scenedesmus obliquus*	Shrinkage of the cell away from the cell wall.	(Santomauro et al. 2012)
3	Ag NMs	6–20	Hoagland's solution	*Spirodela polyrhiza* (aquatic plant)	Affected chloroplast structure.	(Jiang et al. 2014)
4	Ag NMs	15–97	Jaworski's medium (JM)	*Chlorella vulgaris*	Ag NMs were localised in starch granules within the chloroplast of algae.	(Kalman et al. 2015)
5	Ag NMs	20	Talaquil	*Euglena gracilis*	The cells were completely round, compared to the spindle-like morphology of the control cells.	(Li et al. 2015b)
6	Ag NMs	21.2–33.9	OECD 201	*Chlorella pyrenoidosa*	Plasmolysis and membranolysis occurred in cells.	(Zhou et al. 2016)
7	Ag NMs	38–73	Milli-Q water	*E. gracilis*	Ag NMs adsorb only onto the cell surface.	(Yue et al. 2017)
8	TiO_2 NMs	21	f/2 medium (Seawater)	*Chlorella vulgaris*	TiO_2 NMs adhered to the algal cells. Accumulation of TiO_2 NMs by *C. vulgaris*.	(Xia et al. 2018)
9	CeO_2 NMs	82–193	Synthetic freshwater (0.22 µm)	*R. subcapitata*	Presence of CeO_2 is on the outside of *R. subcapitata* cells. CeO_2 was not observed to be internalised within cells.	(Angel et al. 2015)

4.1.6 *Accumulation and transformation of CNTs*

Uptake of SWCNTs and MWCNTs has been demonstrated for a number of aquatic species such as aquatic invertebrates, amphipods, midges, and oligochaetes (Mwangi et al. 2012), earthworms (Petersen et al. 2009b), *Daphnia magna* (Zindler et al. 2016) and microalgae (Long et al. 2012; Youn et al. 2012; Pereira et al. 2014; Zhang et al. 2014; Hu et al. 2015; Pulido-Reyes et al. 2015; Rhiem et al. 2015).

Owing to the propensity of many CNT types (and NMs in general) to agglomerate and settle in aquatic systems, however, there is concern that they might affect benthic organisms and sediment systems more strongly than pelagic species, despite the latter being more frequently studied (Selck et al. 2016). Despite strong evidence for CNT uptake, there is currently limited data available with regard to their potential bioaccumulation (Jackson et al. 2013).

Some recent studies on functionalised and non-functionalised MWCNT accumulation have employed single-celled organisms such as algae (Hu et al. 2016), bacteria and protozoa (Mortimer et al. 2016a). In the case of bacteria and protozoa, an approach employing separation of ^{14}C-labelled functionalised MWCNTs by density gradient centrifugation was described (Mortimer et al. 2016b). The bacterium *Pseudomonas aeruginosa* was found to adsorb the functionalised MWCNTs at concentrations of 0.18 ± 0.04 and 21.9 ± 4.2 μg/mg dry mass at respective nominal concentrations of 0.01 and 1 mg/L. In the same study, an accumulation of up to 0.9 ± 0.3 μg of functionalised MWCNT/mg dry mass was recorded in the protozoan *Tetrahymena thermophila* following trophic transfer via MWCNT-encrusted *Pseudomonas aeruginosa* cells, while up to 3 ± 1 μg of MWCNT dry mass was detected following direct uptake. Thus, protozoa were identified as a potential vector for the transfer of functionalised MWCNTs to the next trophic level (Mortimer et al. 2016a). The alga *Desmodesmus subspicatus* was found to contain mean concentrations of 1.3 ± 0.5, 2 ± 2, and 5 ± 2 μg non-functionalised MWCNTs/mg dry weight following exposure to a suspension of 1 mg/L of MWCNTs for 24, 48, and 72 h respectively (Rhiem et al. 2015).

Daphnia magna has been shown to internalise functionalised MWCNTs. Following a 48 h exposure to a suspension of 0.4 μg/mL, 63 ± 15 μg of the test substance was recorded per mg dry mass (Petersen et al. 2009a). Previously, a value of approximately 29 μg/mg dry mass was reported, following a 48 h exposure of *D. magna* to a 30 μg/L fullerene suspension (Oberdörster et al. 2006).

A recent review by Hu et al. (2016) emphasised the need to consider the complete range of techniques for extraction, isolation, and characterisation of CNTs in order to follow their transformation in natural environments and organisms. However, in the case of CNTs, few data on structural degradation in the aquatic environment have been reported. A strain of the bacterial species *Trabulsiella guamensis* isolated from soil was shown to cause surface oxidation and structural changes in MWCNTs (Chouhan et al. 2016). Furthermore, a lignin peroxidase isolated from the mushroom *Sparassis latifolia* was shown to biodegrade SWCNTs (Chandrasekaran et al. 2014). Current methodologies for isolating CNTs and their transformed forms from complex matrices are still limited, whilst techniques for accurately characterising and quantifying CNTs also remain underdeveloped (Petersen et al. 2009a; Von der Kammer et al. 2012; Petersen and Henry 2012; Pycke et al. 2012; Montaño et al. 2014; Mortimer et al. 2016b). Furthermore, the presence of low CNT concentrations in environmental matrices, and the difficulty in distinguishing natural sources of carbon from those corresponding to CNTs, compound the challenges of separation and analysis. The challenge of determining CNT uptake and accumulation in organisms and environmental samples highlights the necessity of using radiolabelling in laboratory studies (Petersen et al. 2016). In Table 4.2 there is a summary of selected examples showing adsorption/interaction and internalisation of CNTs by microalgae.

Table 4.2: Selected examples showing adsorption/interaction and internalisation of CNTs by microalgae

No.	NMs	Diameter (d) and length (L)	Medium	Microorganism	Additional information	Reference
1	SWCNTs	d: NG L: 300–1200 µm	Gum Arabic solution	*R. subcapitata*	Change in morphology of cell membranes. Reduction in size and deformation of cell membrane.	(Youn et al. 2012)
2	MWCNTs	d: 10–100 nm L: 0.4–4.1 µm	OECD 201	*Chlorella spirulina*	Lost of cellular integrity and cell damage. Cytoplasm outflow onto MWCNT layer. Breakage of cell wall.	(Long et al. 2012)
3	MWCNTs	d: 20–40 nm L: 40–60 µm	Bold's Basal	*Chlorella vulgaris*	Internalisation of the MWCNTs induces membrane rupture and morphological alterations.	(Pereira et al. 2014)
4	SWCNTs	d: 1–2 nm L: 0.5–3 µm	Blue-Green Medium (BG11)	*Chlorella vulgaris*	Plasmolysis. Increases in the number of starch grains and the number of lysosomes. Shrinkage of the cytoplasm as the irregular morphology of the plasma membrane edges.	(Hu et al. 2015)
5	MWCNTs	d: 70 nm L: 3.2 ± 0.9 µm	OECD 201	*Chlorella pyrenoidosa*	Severe plasmolysis was observed for the cells exposed to MWCNTs without DOM and with the two surfactants. Significant cell uptake and internalisation of MWCNTs were observed in the presence of surfactants.	(Zhang et al. 2014)
6	CNTs	d: 5–50 nm	OECD 201	*R. subcapitata*	CNTs attached to the outer algal cell wall. No evidence of internalisation was observed.	(Pulido-Reyes et al. 2015)
7	MWCNTs	d: 5–20 nm L: <1 µm	OECD 201	*D. subspicatus*	Both single tubes and agglomerated CNT material interacted with algal cells. Nanotubes were found attached to the outer cell wall, the inner cell membrane, lying in the cytoplasm and piercing cells.	(Rhiem et al. 2015)

4.1.7 *Aims and objectives of this chapter*

The main scope of this chapter was to investigate the interaction and uptake of NMs with freshwater microalgal *R. subcapitata*, using OECD 201 medium for 72 h, with a major focus on Ag NMs and MWCNTs. The research questions addressed were:

(i) Can Ag NMs and MWCNTs interact with the algal cell wall?

(ii) Can Ag NMs and MWCNTs be internalised into the algal cell?

(iii) What are the effects of Ag NMs and MWCNTs on algal cell structure?

(iv) How do algae differing in their cell walls interact with Ag NMs and MWCNTs?

(v) Is there any effect of sonication method on the NM-induced algal cell structure responses?

The effects of Ag NMs and MWCNTs were examined using the freshwater alga *R. subcapitata* as a model organism (as stated in Chapter 3). To measure algal interaction and internalisation with NMs, the algae were exposed to different concentrations of Ag NMs and MWCNTs for 72 h, based on IC_{50} values obtained from toxicity experiments (see Chapter 3), using bath and probe sonication in OECD 201 medium.

4.2 Methodology

In order to investigate whether the interaction of NMs with algal cells involves internalisation and/or attachment of NMs at the cell surface, the algal cells were fixed and dehydrated in resin for assessment with TEM, or direct samples were taken for analysing with the light microscope. The interaction and uptake of NMs was determined in *R. subcapitata* cells exposed to sublethal concentrations of used NMs for 72 h in OECD 201 medium. The chosen concentration was based on IC_{50} values obtained from the acute toxicity experiments from Chapter 3 (for each NM and sonication type) so as to be able to see the impacts of NMs on algal cell structure without severe damage to the algal cell which could accrue with a high concentration of NMs.

4.2.1 *Algal culture preparation*

The algal culture preparation and exposure experiments for the assays described in this chapter were the same as outlined in Chapter 3 sections 3.2.4 and 3.2.8.

4.2.2 *Algal medium*

OECD 201 medium was prepared as previously described in Chapter 2, section 2.2.2.

4.2.3 *NM stock suspension preparation*

Ag NMs (NM300k) and MWCNTs (NM400) were bath and probe sonicated as previously described in Chapter 2, section 2.2.4.

4.2.4 *Light microscope (LM)*

An LM was used to investigate possible attachment of the NMs with algal cells. The NM concentrations used were as follows: 90 µg Ag/L of bath-sonicated Ag NMs; 190 µg Ag/L of probe-sonicated Ag NMs; 28.5 mg/L of bath-sonicated MWCNT, and 6.83 mg/L of probe-sonicated MWCNT. Algal cells unexposed (control) and exposed (treated) to Ag NMs or MWCNTs were compared after 72 h by light microscopy at 40× magnification. A volume of 3.5 µL droplet of fresh sample was placed on a slide and viewed with a Zeiss Axiophot microscope. Moreover, the cells were examined for possible differences in their cell physiology and agglomeration.

4.2.5 *Transmission electron microscopy (TEM)*

Algae were exposed to different concentrations of Ag NMs and MWCNTs, based on IC_{50} obtained from toxicity experiments (Chapter 3), using bath and probe sonication in OECD 201 medium for 72 h. Briefly, the NM concentrations used were as follows: 90 µg Ag/L of bath-sonicated Ag NMs; 190 µg Ag/L of probe-sonicated Ag NMs; 28.5 mg/L of bath-sonicated MWCNT, and 6.83 mg/L of probe-sonicated MWCNT. TEM was used to investigate possible interaction or/and internalisation of the NMs in algal cells.

After the exposures, cells were centrifuged (2,230 g at 4 °C for 10 min) and washed 3 times with Milli-Q water. Sodium cacodylate buffer (0.1 M) was prepared by dissolving 2.14 g of sodium cacodylate trihydrate (≥98 %) (Sigma Aldrich – UK) in 100 mL of Milli-Q water. Then, glutaraldehyde (3%) was prepared by adding 2.5 mL of glutaraldehyde solution (Sigma Aldrich – UK) in 22.5 mL of sodium cacodylate buffer, and the pH was adjusted to 7.3.

Algal cells were fixed using 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h. Postfixation was carried out in 1% osmium tetroxide in 0.1 M sodium cacodylate for 45 min, then washed 3 times (10 min) in Milli-Q water. These samples were then dehydrated in 50%, 70%, 90% and 100% ethanol (C_2H_5OH) (3 times) for 15 min each, then in two 10-min changes in propylene oxide. Samples were then embedded in TAAB 812 resin.

Sections 1 µm thick were cut on a Leica Ultracut UCT ultramicrotome, stained with Toluidine Blue, and viewed in a light microscope to select suitable areas for investigation. Ultrathin sections 60 nm thick were cut from selected areas, stained in uranyl acetate and lead citrate. The postfixation for TEM sample preparation was carried out at the University of Edinburgh. All TEM images were done on an FEI Tecnai F20 FEG-TEM operated at 200 kV fitted with a Gatan Orius Charge Couple Device (SC600A CCD) camera, at the School of Chemical and Process Engineering, University of Leeds, UK. Figure 4.1 shows a scheme depicting the main steps of the TEM sample-preparation procedures for *R. subcapitata* exposed to NMs for 72 h.

4.2.6 *EDX analysis*

TEM EDX analyses were used to confirm the presence of Ag NMs inside algal cells. The EDX analysis was performed using an Oxford Instruments 80 mm^2 Silicon Drift Detector (SDD) fitted to the TEM and running INCA software (University of Leeds,

UK). However, in terms of MWCNTs, it is difficult to detect MWCNTs through the presence of trace metal contaminants such as Fe, in low concentrations, on the MWCNT walls.

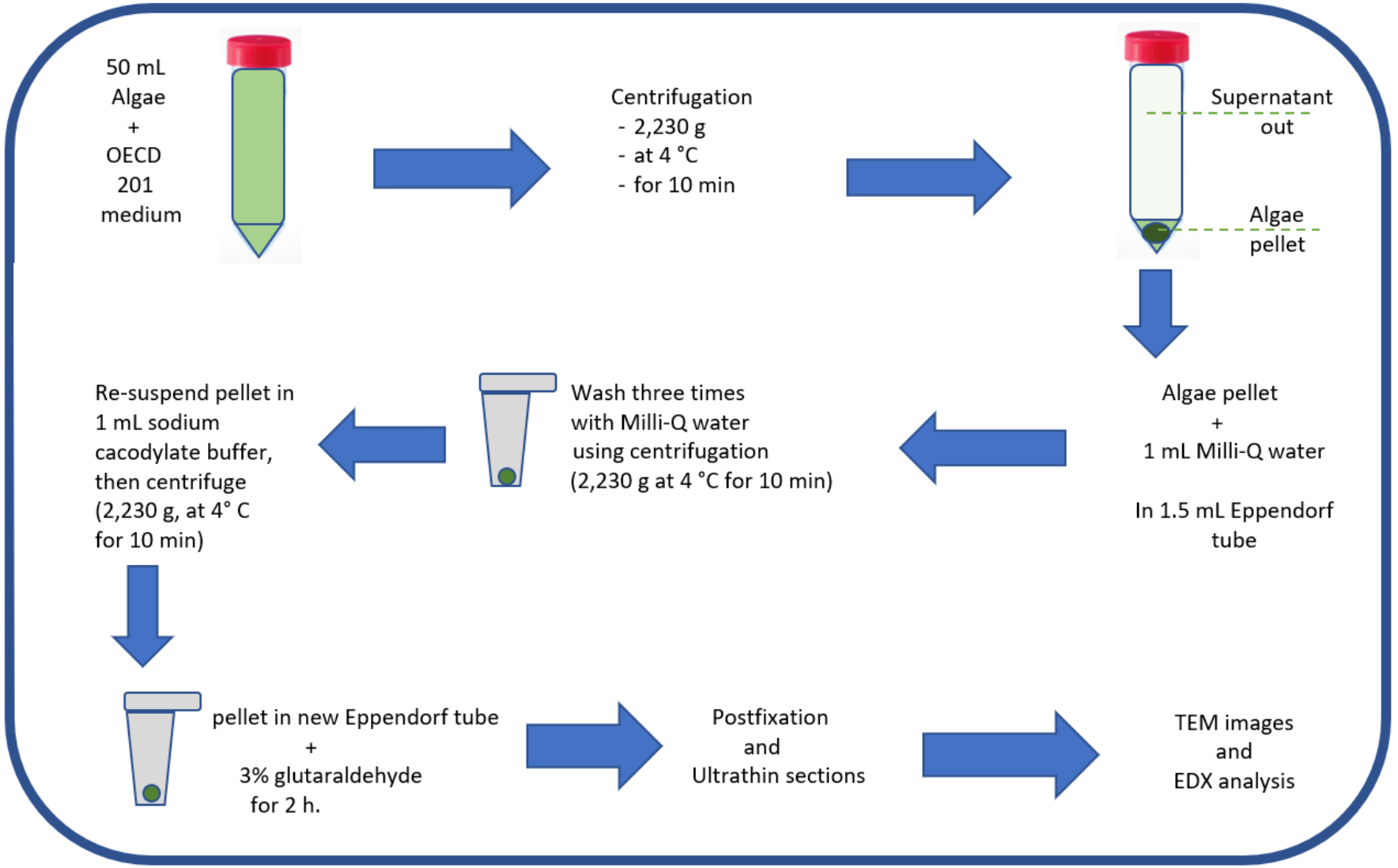


Figure 4.1: Scheme depicting the TEM sample-preparation procedures for *R. subcapitata* exposed to NMs for 72 h

4.3 Results

4.3.1 *Light microscope*

Light microscopical observation was used as an initial method to observe the adherence of NM agglomerates to algal cells and the impact of NMs on algal cell ultrastructure. The chosen NM concentration was decided on with the aim of determining and recognising the impact of NMs on algal cell structure during the exposure time without damaging or completely destroying algal cells. LM analysis revealed that in the control (untreated) exposure, *R. subcapitata* cells have a nice helical shape and were distributed throughout the OECD 201 medium (Figure 4.2 A and B).

The results of this study showed that exposure to Ag NMs and MWCNTs led to an observable alteration in *R. subcapitata* cell shape. When subjected to 90 µg Ag/L of bath-sonicated Ag NMs for 72 h and exposed to 190 µg Ag/L of probe-sonicated Ag NMs for 72 h, the algal cells became more uneven and thinner in both exposure scenarios compared to untreated cells. Moreover, the *R. subcapitata* cells lost their helical shape (Figure 4.2 C and D).

In terms of MWCNTs, no huge difference in algal cell morphology was observed after exposure to bath- and probe-sonicated MWCNT suspensions. Moreover, in both cases most algal biomass was trapped in the solid mass of MWCNT agglomerates, with some free algal cells separated from the main cluster. However, some free algal cells were thinner and lost their unique shape compared to control cells (Figure 4.2 E and F).

The sample content affected the image background. For example, in the control samples, the background was green, which was indicative of the green colour of the algae. However, the presence of polyoxyethylene glycerol trioleate and polyoxyethylene (20) in Ag NMs gave a yellowish colour. Moreover, the black colour of MWCNTs does not reflect light, which makes the algae images much better than other cases.

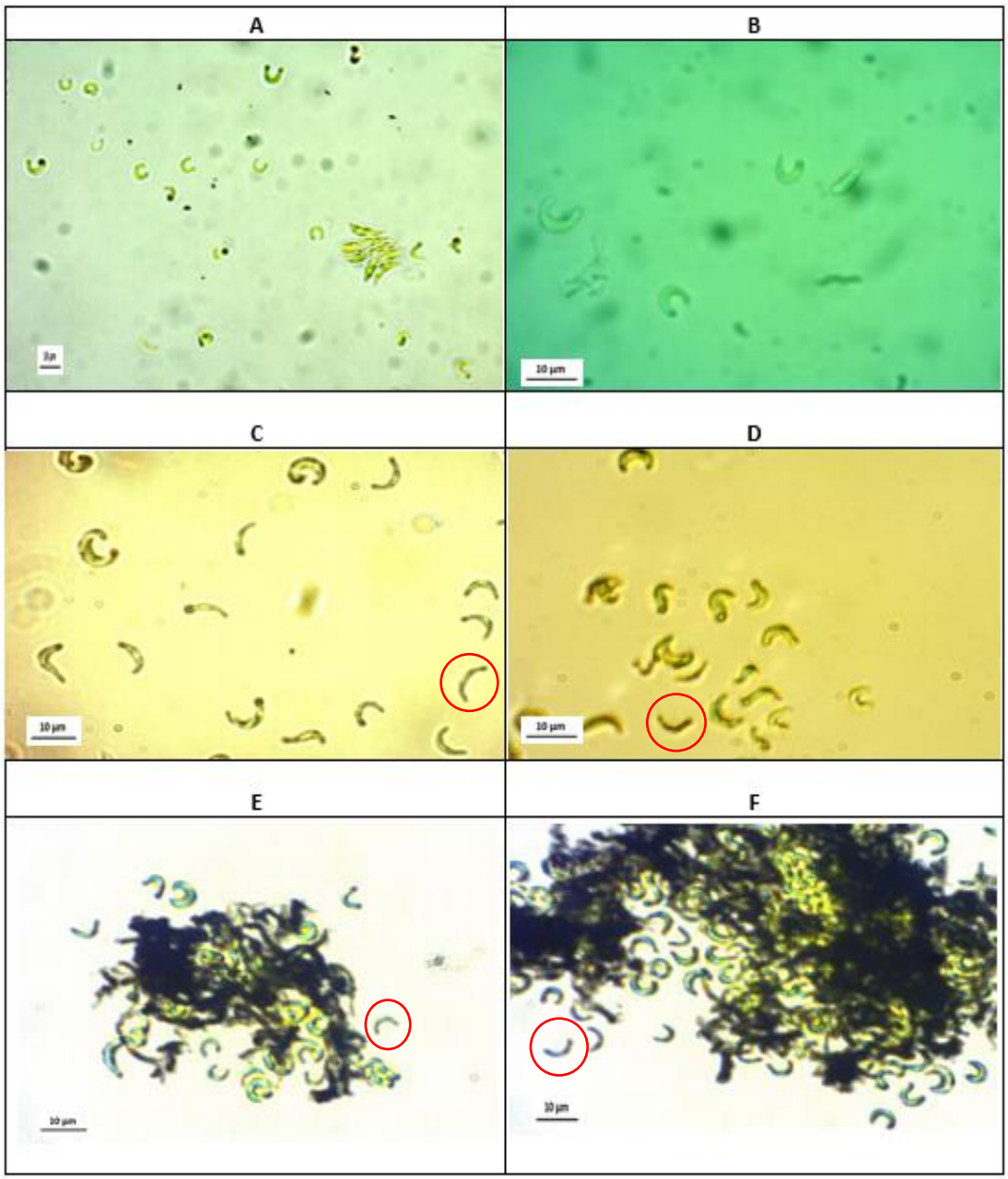
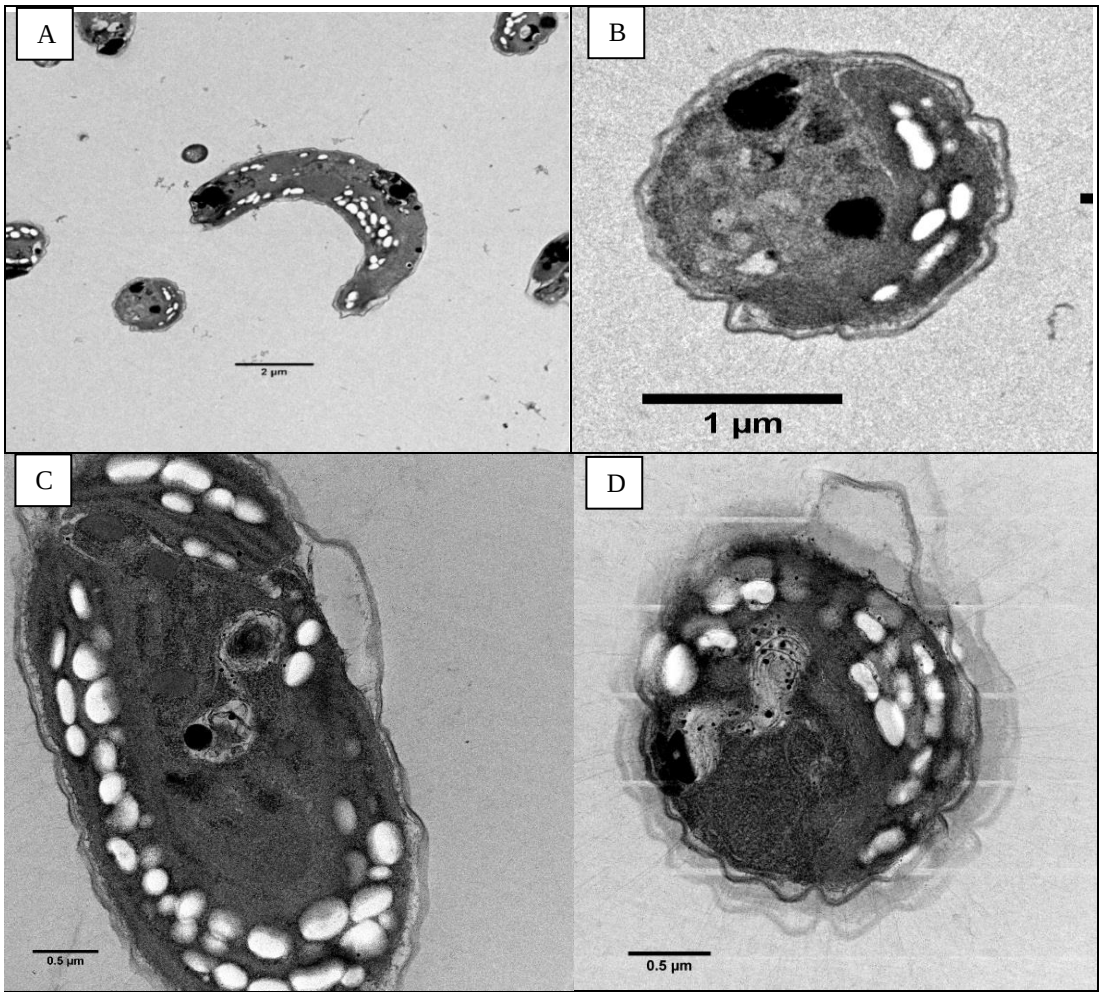


Figure 4.2: Light microscopic images of *R. subcapitata* cells in OECD 201 medium, taken at 40× magnification. A and B: *R. subcapitata* cells in control samples. C: Algal cells exposed to 90µg Ag/L of bath-sonicated Ag NMs for 72 h. D: Algal cells exposed to 190 µg Ag/L of probe-sonicated Ag NMs for 72 h. E: Algal cells exposed to 28.5 mg/L of bath-sonicated MWCNTs for 72 h. F: Algal cells exposed to 6.83 mg/L of probe-sonicated MWCNTs for 72 h. Red circles indicate some algal cells were thinner and lost their helical shape. Scale bar 10 µm.

4.3.2 *TEM*

Further TEM analysis of *R. subcapitata* introduced to bath- and probe-sonicated suspensions of Ag NMs and MWCNTs confirmed microscopic observations. All results are shown in Figure 4.3. Untreated cells of *R. subcapitata* with a helical shape are shown in Figure 4.3 A (whole cell), and B (cross section). Exposure to 90 µg Ag/L of bath-sonicated Ag NMs for 72 h (Figure 4.3 C) and to 190 µg Ag/L of probe-sonicated Ag NMs for 72 h in OECD 201 medium (Figure 4.3 E) led to cell shrinkage. *R. subcapitata* cell morphology was altered by MWCNT suspensions (bath- and probe-sonicated suspensions) in comparison to Ag NMs (Figure 4.3 G and I). Figure 4.3 G, I and J show the significantly altered shape of algal cells. In addition, cross-section pictures suggest the disintegration of the cell wall. Furthermore, probe-sonicated MWCNTs had a huge effect and resulted in damage to the algal cell structure (Figure 4.3 J).



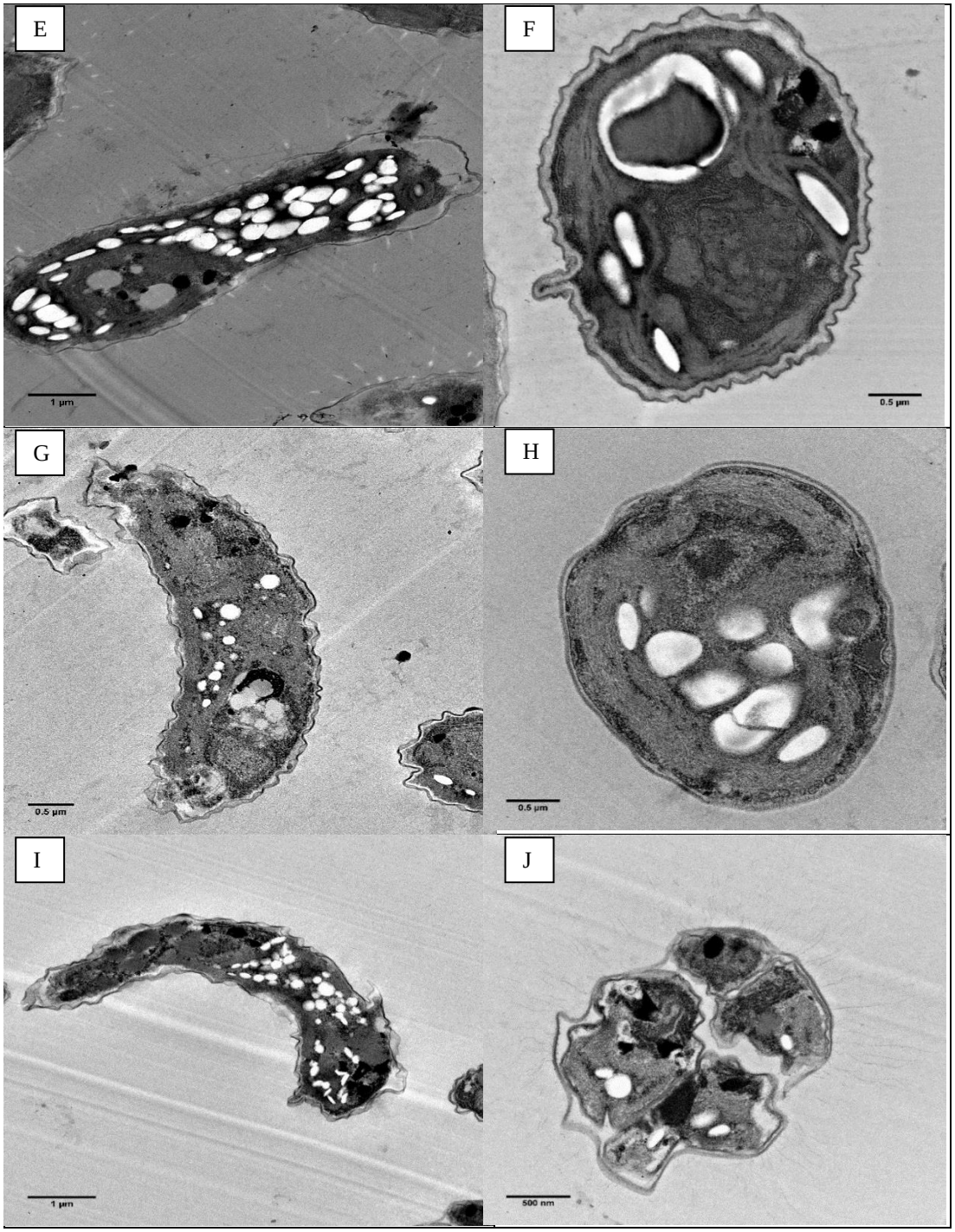
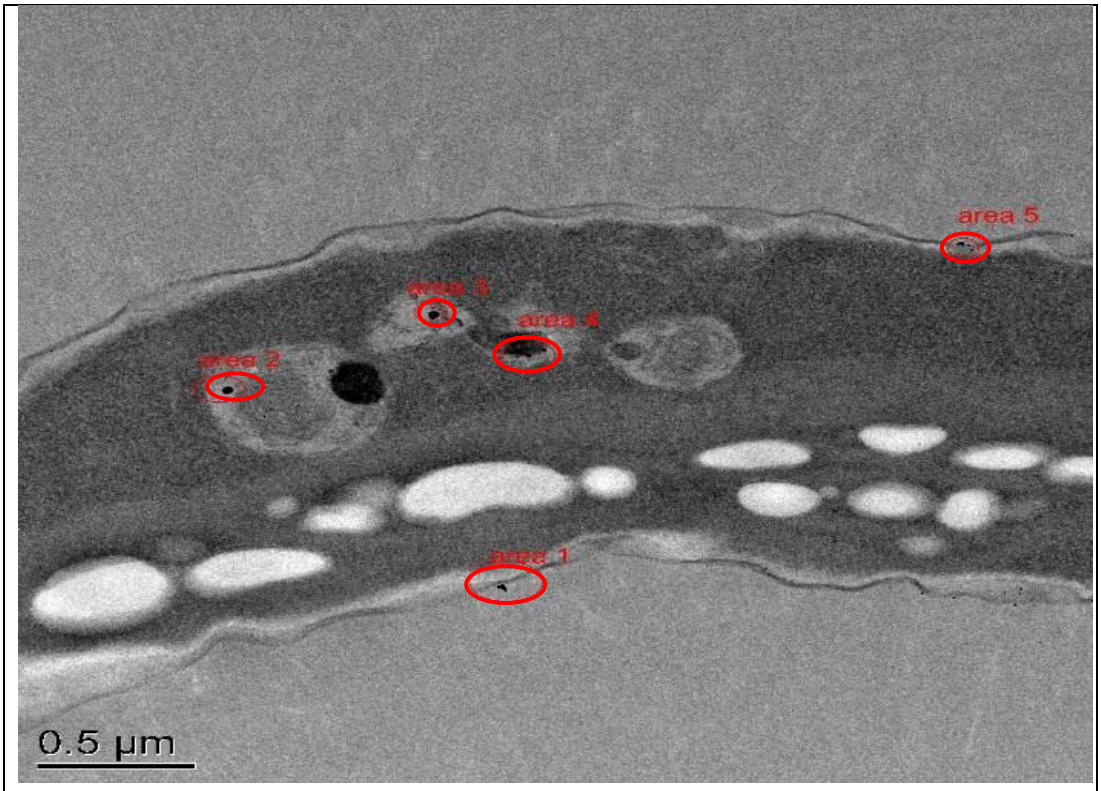


Figure 4.3: Selected transmission electron microscopy (TEM) morphology micrographs of *R. subcapitata* after algae were treated for 72 h in OECD 201 medium. A and B: control (non-exposed); C and D: exposed to 90 µg Ag/L Ag NMs (bath sonication); E and F: exposed to 190 µg Ag/L Ag NMs (probe sonication); G and H: exposed to 28.5 mg/L MWCNT (bath sonication); I and J: exposed to 6.83 mg/L MWCNT (probe sonication). Shrinkage of the cell away from the cell wall can be observed with bath- and probe-sonicated Ag NMs in C, D and E. Cell deformation and an irregular shape of *R. subcapitata* post exposure to probe-sonicated MWCNTs is shown in I and J.

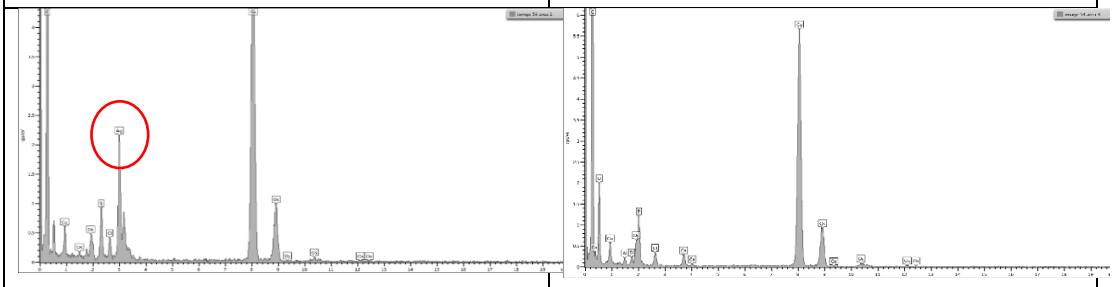
4.3.3 *EDX analysis*

To investigate the cellular uptake of Ag NMs by *R. subcapitata,* TEM analysis coupled with a chemical microanalysis EDX was used. Due to the nature of MWCNTs (carbon-based NMs), the EDX technique is not a suitable method to investigate cellular uptake of MWCNTs by *R. subcapitata,* therefore TEM analyses were performed to investigate the interactions between MWCNTs and algal cells. Figure 4.4 presents images of *R. subcapitata* exposed to bath-sonicated Ag NMs at 90 µg/L. Red circles indicate Ag NM signals. EDX analyses showed no evidence of internalisation of Ag NMs into the algal cells, however an uptake of Ag NMs on the algal cell wall was observed. The Cu signal is from the grid and the Os, U and Pb signals are due to fixation.



Area 1: Ag NM interaction on algal cell wall

Representative areas 2, 3 and 4: there are no detectable Ag NMs inside algal cell



Area 5: Ag NM interaction on algae cell wall

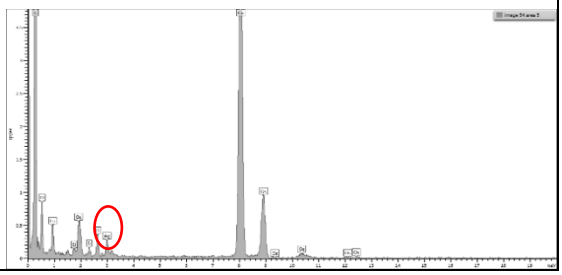
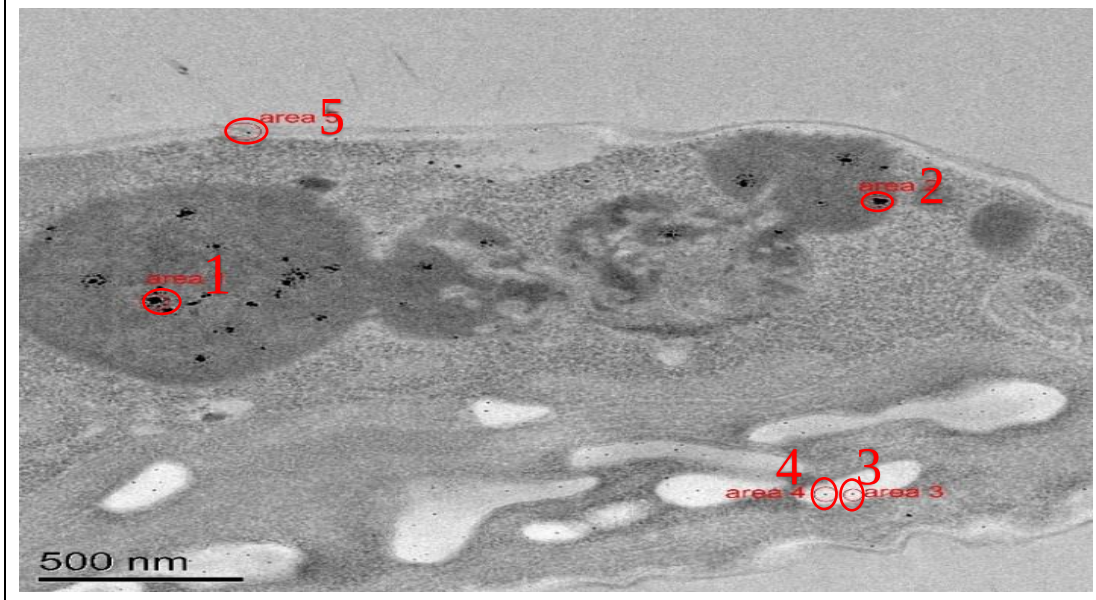
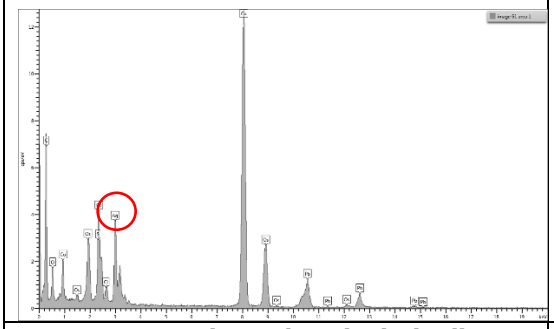


Figure 4.4: Transmission electron microscopy (TEM) observations of ultrathin slices of *R. subcapitata* after algae were exposed to 90 µg/L of bath-sonicated Ag NMs for 72 h in OECD 201 medium. Energy-dispersive X-ray (EDX) spectra show Ag peaks for Ag NMs on the algal cell wall. Red circles indicate areas analysed for Ag NM signals and the labels for Ag NM peaks.

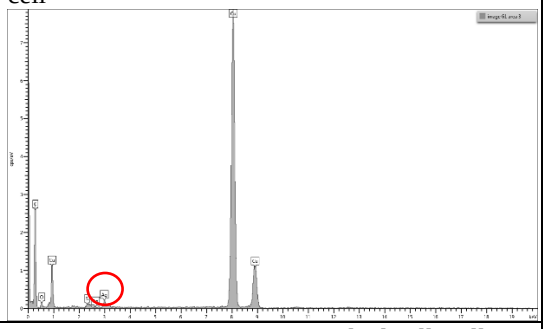
In contrast, by using different types of sonication methods for NMs, Figure 4.5 presents an image of *R. subcapitata* treated with probe-sonicated Ag NMs at 190 µg/L. Red circles indicate areas analysed for Ag NM signals. EDX analyses suggest evidence of internalisation of Ag NMs into the algal cell, as well as their presence on the cell wall. In addition, Ag NMs were visualised externally.



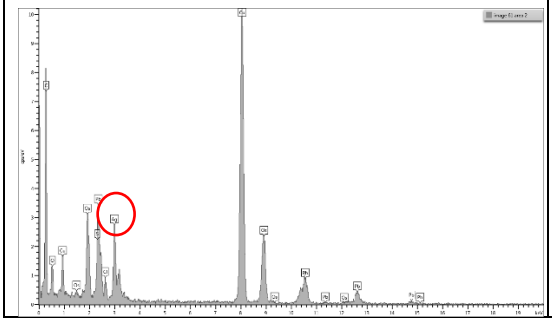
Area 1: Ag NMs detected inside algal cell



Areas 3 and 4: Ag NMs detected inside algal cell



Area 2: Ag NMs detected inside algal cell



Area 5: Ag NM interaction on algal cell wall.

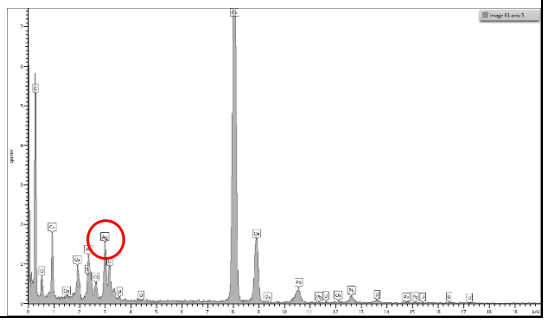


Figure 4.5: Transmission electron microscopy (TEM) observations of ultrathin slices of *R. subcapitata* after algae were exposed to 190 µg/L of probe-sonicated Ag NMs for 72 h in OECD 201 medium. Energy-dispersive X-ray (EDX) spectra show Ag peaks for Ag NMs on the algal cell wall as well as inside the algal cell. Red circles indicate areas analysed for Ag NM signals and the labels for Ag NM peaks.

In terms of MWCNTs, Figure 4.6 presents an image of *R. subcapitata* exposed to bath-sonicated MWCNTs at 28.5 mg/L. TEM analyses showed no evidence of internalisation of MWCNTs into the algal cell. MWCNTs were visualised externally. Moreover, large agglomerates outside the cells, not attached to the cell surface, were visualised.

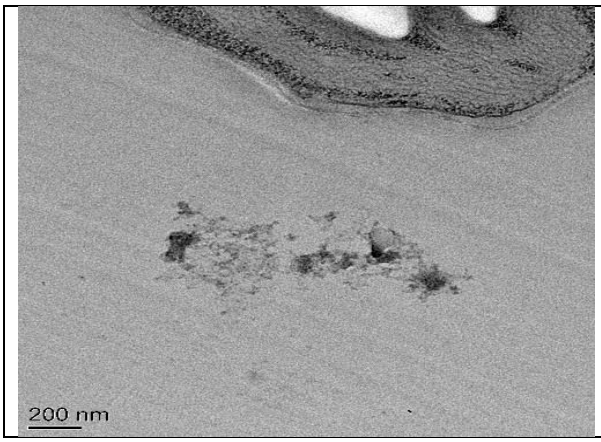


Figure 4.6: Selected transmission electron microscopy (TEM) micrograph of *R. subcapitata* after exposure to 28.5 mg/L MWCNT (bath sonication) for 72 h in OECD 201 medium.

Regarding probe-sonicated MWCNTs, Figure 4.7 presents images of *R. subcapitata* exposed to probe-sonicated MWCNTs at 6.83 mg/L. TEM analyses showed no evidence of internalisation of MWCNTs into the algal cell. Moreover, large agglomerates outside the cells, and one tube was attached to the cell surface, were visualised. Furthermore, exposure to MWCNTs altered the shape of algal cells.

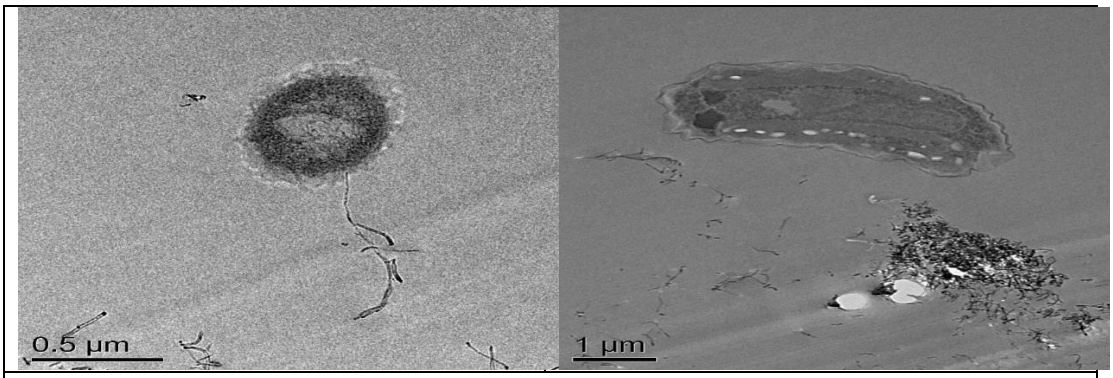


Figure 4.7: Selected transmission electron microscopy (TEM) micrographs of *R. subcapitata* after exposure to 6.83 mg/L of MWCNT (probe sonication) for 72 h in OECD 201 medium.

4.4 Discussion

LM, TEM analysis and EDX were used to investigate the interactions of Ag NMs and MWCNTs with *R. subcapitata* cells. Moreover, algal cell morphology (e.g. damage to the algal cell structure), NM uptake and internalisation were investigated through the exposure to NMs (Ag NMs and MWCNTs). LM results demonstrated that *R. subcapitata* subjected to all tested NM suspensions responded by becoming thinner and losing their unique shape compared to control cells. Moreover, *R. subcapitata* cell morphology was thinned more, and more damaged, by probe-sonicated NMs compared to bath-sonicated NMs.

The agglomeration of MWCNTs resulted in shading on algal cells which led to reduced light transmittance and reduced viability of phototrophic microorganisms (see section 4.3.1). These findings are consistent with data obtained by Schwab et al. (2011) using *R. subcapitata* exposed to MWCNTs prepared by ultrasonication in the presence of NOM. Moreover, Schwab and colleagues confirmed that growth inhibition was correlated strongly with both algal cells in MWCNT agglomerates and shading. In contrast, by using metal-based NMs, Aruoja et al. (2009) suggested that the shading of light by CuO, ZnO and TiO_2 NMs was not contributing to the overall toxic effect towards *R. subcapitata*. Moreover, CNTs accumulated on the microalgal surface could inhibit algal growth by enhanced shading and agglomeration effects (Schwab et al. 2011). Furthermore, microorganism-adsorbed NMs were also observed to result in toxicity by pitting the cell walls (Choi et al. 2008), inducing intracellular leakage via damaged cell walls (Cherchi et al. 2011; Dalai et al. 2012). TEM images for *R. subcapitata* exposed to bath- and probe-sonicated Ag NMs are in agreement with those obtained by Pakrashi et al. (2013). Their findings confirm that *Chlorella ellipsoidea* exposed to aluminium oxide NMs (Al_2O_3 NMs) have an effect on cell structure and distortions in morphology (Pakrashi et al. 2013).

TEM results confirmed all the statements above. In addition, the internalisation of NMs within algal cells was analysed. TEM analyses showed no evidence of internalisation of MWCNTs (bath or probe sonicated) into the algal cell. This could be due to the lack of sufficient contrast to distinguish between carbon-based background cell components and the CNTs.

Interestingly, EDX analyses showed evidence of internalisation of Ag NMs coming from probe-sonicated suspensions into the algal cells and no evidence of Ag NMs coming from bath-sonicated suspensions into the algal cells and hardily to find.

Ag NMs were visualised externally. Furthermore, large MWCNT agglomerates outside the cells, not attached to the cell surface, were visualised when using bath sonication. However, when using probe sonication, some CNTs were noticed attached to the algal cell surface. Furthermore, a look at cells exposed to the previously identified toxic combination of probe-sonicated MWCNTs in comparison with cells from untreated control samples showed significant changes to the morphology of cell wall.

In these dividing cells, NMs-treated cells have reduced size and deformed cell walls. These impacts could be attributable to the destructive effects of ROS due to the fact that membrane and wall integrity are a primary target (Cabiscol et al. 2000), but one may not exclude the potential physical interaction between cells and MWCNTs, even though the TEM images do not show such occurrences. A previous study on SWCNT–organism interactions showed that cell death could be attributed to the physical piercing of cell membranes by individual SWCNTs in well-dispersed suspensions containing high concentrations up to 50 µg/g (Kang et al. 2007).

This study focused on understanding the interaction of NMs with *R. subcapitata* cells and the uptake of Ag NMs and MWCNTs by comparing exposures to bath- and probe-sonicated NM suspensions. In order to better understand how the sonication method could influence the uptake of Ag NMs, TEM analyses coupled with EDX were performed. In this study, evidence of internalisation of Ag NMs into the *R. subcapitata* cells was shown for Ag NM suspensions dispersed using probe-sonication methods. It might suggest the difference in agglomerate sizes to primary particle size. Most likely, probe-sonicated Ag NMs were smaller in size in comparison with bath-sonicated suspensions. Given that algal cell walls are often porous in their structure (usually between 5 and 20 nm), and their permeability changes during mitosis, NMs less than 20 nm in size may pass freely through the cell wall. Moreover, NMs may induce the formation of larger new pores permeable to bigger NMs (Navarro et al. 2008b), which might be the case in the present study using *R. subcapitata* as a test microorganism. Furthermore, there is a possibility that such a high concentration of Ag NMs may lead to an increase in cell wall permeability and subsequent internalisation of NMs. Based on the finding in this research study, the change in cell structure and decrease in osmotic pressure were probably due to damage to the cell wall mediated by ROS production (see Chapter 5).

In terms of NM uptake into the microalgal surface, specific interactions may occur between the surface of the NMs and microorganisms. Schwab et al. (2011) indicated that hydrogen bonds between the algal cell surfaces and oxygen defects of CNTs might form to coagulate them. In addition, the majority of metal-based NMs can likely release metal ions that may bind with cationic and anionic exchange sites on the algal cell surfaces, and thus provide binding sites for the NMs (Vinopal et al. 2007; Khan et al. 2011; Chen et al. 2012b).

The adsorption of NMs on microorganisms might result in a series of subsequent effects from their toxicity, such as physical damage and biochemical injury (Thill et al. 2006; Choi et al. 2008; Wei et al. 2010a; Dalai et al. 2012). The findings in this research study confirm the adsorption of bath- and probe-sonicated Ag NMs on the algal cell wall. These results are along the lines of those obtained throughout the TEM images using *R. subcapitata* exposed to Ag NMs, as the agglomerates varying in size could be visualised externally (Ribeiro et al. 2015).

Furthermore, the change in cell structure can be potentially related to a decrease in the cellular turgor pressure likely resulting from the structural wall and membrane damage mediated by the ROS production. This hypothesis will be investigated in the next chapter.

The internalisation of Ag ions into algal cells can be explained by one of the following three possible mechanisms (Campbell 1995; Campbell et al. 2002). First, Ag uptake generally occurs via accidental cation transport or transport through a system used for the uptake of other essential cations (e.g. K, Na), as Ag is not an essential element for algal growth. Second, Ag ions may cross cell membranes via passive diffusion directly through the lipid bilayer (i.e. as a neutral $AgCl_0$ complex). Finally, Ag ion uptake may occur via accidental anion transport or transport via anion transporters as silver-anion complexes (e.g. $AgCl^-$) (Campbell 1995; Campbell et al. 2002; Lee et al. 2004).

In green microalgae, Se can be transported into the cell as anionic macronutrients via a transport system but with low affinity (Neumann et al. 2003; Sun et al. 2014). Some algae showed growth stimulation at lower Se levels within a defined range, but almost all were inhibited at higher concentrations (Kiffney and Knight 1990; Douskova et al. 2007). Some studies have already revealed Se is needed as a nutrient of algae in the synthesis of proteins and lipids to enhance cellular division (Furness and Rainbow 1990).

In terms of NMs, there are some possible subcellular uptake or accumulation pathways of insoluble NMs across the particle barriers of plant and algal cells. Schwab et al. (2016) lists the main features of NM transport to and through the cell surface as follows: passively diffusing through permeable regions of the cuticle and cell wall pores; facilitated transport depending on surface properties; facilitated transport by natural organic matter (humic acids, etc.) and ubiquitous interactions with microorganisms.

Ag NMs might be internalised into algal cells through the pores to cross the algal cell wall, particularly NMs with a diameter smaller than 20 nm (Moore 2006; Domingo et al. 2019). The findings in this research study are in agreement with those obtained by Kalman et al. (2015). Kalman and colleagues investigated the bioaccumulation dynamics of three differently coated Ag NMs and aqueous Ag in a simple freshwater food chain. In this study, Ag NMs were localised in starch granules within the chloroplast of *C. vulgaris* as confirmed by TEM images. They suggested that granules might work as a storage site for NMs in *C. vulgaris*.

The intracellular uptake of Ag NMs has been observed in the freshwater alga *Ochromonas danica* by Miao et al. (2010); however, it is unclear whether growth was inhibited directly (by Ag NMs inside the cells) or indirectly (by the release of Ag^+ internally). Moreover, the internalisation of NMs in *O. danica* was observed by TEM in cells from treatments with no obvious toxic effects. Despite carrying out specific membrane permeability assessments, the authors excluded the possibility that an increase in cell membrane permeability resulted in a passive uptake of NMs. However, polymer-coated CuO NMs (with an average size of 65 nm) were able to penetrate the cell of *Chlamydomonas reinhardtii* in particulate form after 6 h of exposure, as observed by Perreault et al. (2012). Internalisation of CuO NMs (<5 nm) was also revealed in the prokaryotic alga *Microcystis aeruginosa* by Wang et al. (2011) with no increase in membrane permeability observed during 24 h exposure, the time during which internalisation could have taken place.

In TEM images, it can be noticed the white circles in *R. subcapitata* indicate the stress response, showing an increase in the number of starch granules due to Ag NMs and MWCNT exposure. Zhou et al. (2012) observed an increased number of starch granules in *Chlorella pyrenoidosa* after exposure to dissolved zinc and copper. Their findings indicate that the freshwater microalgae (*C. pyrenoidosa*) developed a defence mechanism against such ionic metals, and this defence mechanism likely isolated the

toxicants. There is another possible mechanism that can protect microorganisms against metal toxicity, which is the secretion of exopolymeric substances (EPS). EPS can either stabilise NM dispersions or induce their agglomeration, and thus may either exacerbate or reduce the direct toxicity of NMs (Wilkinson and Reinhardt 2005). Furthermore, EPS can also provide abundant binding ligands for the toxicants (such as metal ions) released from NMs, reduce their accumulation or change their subcellular distribution in the cells and consequently influence the indirect effects of NMs (McConville et al. 1999; Pistocchi et al. 2000; Salgado et al. 2005; Haye et al. 2006; Alderkamp et al. 2007).

The external surface of *C. vulgaris* contains EPS, proteins and carbohydrates, which facilitate the binding of metal ions (Kuyucak and Volesky 1990). The binding with metal ions can lead to agglomerates of these substances with metal ions and reduce the direct toxicity of NMs. In terms of Ag^+, Miao et al. (2009) for instance, found a higher Ag^+ tolerance due to the secretion of EPS by the marine diatom *Thalassiosira weissflogii*, suggesting that EPS may provide binding ligands for toxicants released from NMs.

4.5 Conclusion

Under the tested conditions in the present study, the obtained findings confirm that exposure to Ag NMs and MWCNTs affects algal growth and cell morphology. Bath- and probe-sonicated Ag NMs interacted with the *R. subcapitata* cell wall and affected cell structure. *R. subcapitata* lost their unique cell structure after exposure to Ag NMs and MWCNTs using both types of sonication.

The MWCNT mode of interaction with the algal cells was by agglomeration of the algal cells, choking them. Moreover, the probe-sonicated MWCNTs had a huge effect on cell structure compared to suspension prepared by bath sonication. Furthermore, the dark physical appearance of MWCNTs contributed to algal growth inhibition.

The internalisation of Ag NMs into the algal cells seemed to occur only with suspension prepared using probe sonication, and hardly with bath sonication, probably owing to increased membrane permeability. However, the interaction of Ag NMs was observed and placed on algal cell wall by using both types of sonication (bath and probe). Finally, MWCNTs did not display confirmed internalisation into *R. subcapitata*.

Chapter 5

Role of oxidative stress in NM toxicity to algae

Chapter 5 **Role of oxidative stress in NM toxicity to algae**

5.1 Introduction

Aquatic organisms such as microalgae are widely used in toxicology and ecotoxicology due to their sensitivity, ubiquity and simplicity of cultivation. Any processes and substances that affect the microalgal population can have consequences for higher trophic levels. It is important to understand the mechanism of toxicity of substances in order to identify the biomarkers of toxicity.

5.1.1 *Oxidative stress*

Reactive oxygen species (ROS) exhibit both beneficial and harmful effects in organisms. They are produced during the metabolism of oxygen, as well as taken up from the extracellular environment (Martindale and Holbrook 2002). The most common toxicity pathway for NMs in algae is through the stimulation of oxidative stress (Nel et al. 2006; Bystrzejewska-Piotrowska et al. 2009; Wiesner et al. 2009), which has been observed to be responsible for NM toxicity in algae (Farré et al. 2009; Quigg et al. 2013; Saxena 2018). Oxidative stress is defined as a disturbance in the stability of production of ROS and antioxidant defences, which may lead to cell injury or damage (Betteridge 2000; Johnston et al. 2018). ROS can be defined as "molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals" (Valko et al. 2007). In other words, the unpaired electron can make the molecular fragments highly reactive (Nel et al. 2006). These molecules are known to cause oxidative damage to protein, lipids and DNA following environmental stress, where ROS levels are usually elevated (Pan et al. 2011; Walters et al. 2014).

ROS can contribute to important physiological functions in the body. However, they can also attack biological molecules (lipids, proteins, DNA) and compromise their normal function, such as in lipid peroxidation and damage to proteins, lipids and DNA, leading to cell damage (Valko et al. 2007; Gorokhova et al. 2013; Johnston et al. 2018). ROS include free radicals, for example superoxide ($O_2^{-\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals, or nonradical oxygen species such as hydrogen peroxide (H_2O_2). The electrons produced interact with oxygen and the highly reactive superoxide anion is formed. The cells respond to the superoxide anion by producing superoxide dismutase, which converts the $O_2^{-\bullet}$ to hydrogen peroxide. The hydrogen peroxide is decomposed enzymatically to water and oxygen by glutathione (GSH) and catalase (Singh et al.

2009; Shvedova et al. 2012). At the same time, the antioxidant defence system, for example superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), changes following ROS generation to maintain the pro-oxidation/anti-oxidation balance (Li et al. 2015a). The excessive production of ROS in cells can overwhelm the natural antioxidant system, leading to oxidative stress. Oxidative stress represents an imbalance in the dynamic equilibrium between antioxidant defence mechanisms and ROS, and this imbalance can lead to toxicological outcomes (Singh et al. 2009).

NMs such as Ag NMs (Oukarroum et al. 2012a; Qian et al. 2016), CNT (Wei et al. 2010b; Nogueira et al. 2015; Thakkar et al. 2016), TiO_2 NMs (Dalai et al. 2013), CuO NMs (Melegari et al. 2013), Cr_2O_3 NMs (Costa et al. 2016) and Fe NMs (Bhuvaneshwari et al. 2017) have been reported to induce the generation of reactive oxygen species in algal cells.

5.1.2 *Cellular antioxidant defence in algae*

The antioxidant is an enzymatic and non-enzymatic defence system that can provide protection against harmful metal-mediated free radical attacks (Valko et al. 2007; Augoulea et al. 2009). Algal tolerance for metal pollution partly depends on its defence responses to prevent oxidative damage (Pinto et al. 2003). The change in the antioxidant defence system can be measured with SOD, CAT and GSH levels as biomarkers (Li et al. 2016). SOD is an enzymatic antioxidant that can scavenge superoxide anions generated by metal-based stress. SOD is the cell's first line of defence against ROS, which catalyses the disproportionation of $O_2^{\bullet-}$ to O_2 and H_2O_2 (Hassan and Scandalios 1990; Ken et al. 2005).

CAT is a ubiquitous antioxidant enzyme, which is present in most aerobic cells. CAT is involved in the detoxification of hydrogen peroxide (H_2O_2) and ROS. The CAT enzyme can help to catalyse the production of H_2O from H_2O_2 inside the cells through catalysing the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water (catalytic activity) (Torres et al. 2008; Cayman 2019a). Furthermore, SOD and CAT are two important antioxidant defence enzymes that are capable of eliminating overproduced ROS in phytoplankton (Li et al. 2019). GSH is an antioxidant and major free radical remover. The role of GSH as a reductant is extremely important, particularly in the highly oxidising environment of photosynthetic cells. Moreover, algae can respond to metal-based stress by increasing the GSH concentration (Li et al. 2016).

5.1.3 *Effect of NMs on ROS production in algae*

There are relatively few toxicological studies that have investigated the impact of NMs on oxidative stress in algae (Moos and Slaveykova 2014; Pikula et al. 2019). A summary of existing studies which have investigated the stimulation of oxidative stress in algae is provided below. When possible, details of the sonication method are provided; if they are not provided, this is because they were not clearly communicated in the scientific paper.

Oukarroum et al. (2012a) assessed the inhibitory effects of Ag NMs (0.01–10 mg/L) in two species of green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*, 24 h post exposure. Ag NM suspensions were probe sonicated before use for 2 min. ROS generation was measured using the cell-permeable indicator 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). Their study indicates that exposure of both algal species to Ag NMs induced an increase in intracellular ROS production. ROS formation increased by 44% and 123% compared to control (p<0.05) at 1 mg/L of Ag NMs in *Chlorella vulgaris* and *Dunaliella tertiolecta*, respectively. In addition, it was observed that high levels of ROS induction can lead to cell structure damage (Oukarroum et al. 2012a).

Dalai et al. (2013) assessed whether TiO_2 NMs (0.05–1 mg/L) induced ROS production in the freshwater algal *Scenedesmus obliquus* up to 72 h post exposure. TiO_2 NM suspensions were dispersed using a probe sonicator for 5 min. The generation of intracellular ROS was quantified using a DCF assay. A significant increase in the ROS production (by 15.24% and 20.39%) was observed in the treated algal cells at 1 mg/L after 24 and 72 h, respectively, as compared to the control.

Similarly, Ji et al. (2011) investigated the toxicity of 5–1,000 mg/L of TiO_2 NM to the green algae *Chlorella sp*. TiO_2 NM suspensions were shaken for 2 days, then centrifuged at 1,352 g for 30 min. The results showed that agglomerated TiO_2 NMs caused cellular damage along with ROS generation in algal cells (*Chlorella sp*.).

Costa et al. (2016) investigated the effect of chromium oxide (III) NMs (Cr_2O_3 NMs) on the production of ROS and in the microgreen alga *Chlamydomonas reinhardtii*. Cr_2O_3 NM suspensions were dispersed for 4 min using an ultrasonic probe sonicator at 50% power amplitude. *Chlamydomonas reinhardtii* cells were exposed to Cr_2O_3 NMs (10, 100, 1,000 and 10,000 mg/L) for a period of 72 h. The intracellular ROS level was determined using the fluorescent dye 2′7′-dichlorodihydrofluorescein diacetate. It was demonstrated that ROS levels increased by 38.5%, 63.39% and

66.81% at Cr_2O_3 NM concentrations of 100, 1,000 and 10,000 mg/L, respectively, compared to the control.

Bhuvaneshwari et al. (2017) aimed to determine the contribution of oxidative stress to the toxicity of chemically and biologically synthesised zero-valent iron NMs (Fe NMs) in the freshwater green algae *Scenedesmus sp*. Fe NM suspensions were centrifuged at 112 rpm for 30 min. A DCF assay was used to assess the oxidative stress induced by Fe NMs in *Scenedesmus sp*. ROS generation was increased by 13.6%, 32.2% and 39.4% after algae were exposed to 1,000 mg/L of Fe NMs at 24, 48 and 72 h, respectively.

Long et al. (2012) investigated the mechanism of CNT toxicity towards the microalgae *Chlorella sp*. MWCNTs were bath sonicated for 15 min. The length of the MWCNTs was 0.8–3.2 μm, and their diameter was in the 9–70 nm range. The formation of intracellular ROS was determined using H_2DCFDA. The results indicate that the effects of MWCNTs on tested algae can be clarify by the combined effects of agglomeration, shading effects and oxidative stress with the quantitative contributions from these mechanisms depending on the MWCNT size and concentration. It was identified that 96 h post exposure to the MWCNT at IC_{50}, oxidative stress accounted for approximately 50% of the algal growth inhibition (Long et al. 2012).

Nogueira et al. (2015) investigated the effects of graphene oxide (GO) NMs (0.5–100 mg/L) on microalgae *R. subcapitata* 96 h post exposure in a growth chamber with illumination on a 12:12 h light–dark cycle. GO suspensions were probe sonicated at 30% power amplitude and pulse cycles of 15 s on and 5 s off for 4 h, then centrifuged at 21,000 g for 60 min. Oxidative stress was evaluated based on a DCF assay and using a flow cytometer equipped with an argon-ion excitation wavelength (488 nm). Results indicate a significant difference in the levels of oxidative stress and membrane damage for concentrations starting at 10 mg/L. Results from this investigation highlighted a positive correlation between oxidative stress and membrane damage for GO concentrations higher than 10 mg/L.

Overall, the results of the outlined studies demonstrate that different types of NMs can cause ROS production in algal species. There were different approaches used to assess ROS production induced by NMs in algal species, such as DCF assays, plate reading and flow cytometry. In addition, it is observed that the experimental model (including species used, concentration and exposure method) had an impact on the level of ROS generation, which complicates comparisons between different investigations.

Furthermore, the most common results were lipid peroxidation, membrane damage and decrease of cell viability.

5.1.4 *Effect of NMs on antioxidant mechanisms in algae*

Investigations that assess the effect of NMs on antioxidants have used a variety of models including microalgae. In a study which set out to determine the photosynthetic toxicity and oxidative damage induced by Fe_3O_4 NMs on *Chlorella vulgaris*, Chen and colleagues (2012c) added an appropriate amount of Fe_3O_4 NM powders to the *C. vulgaris* culture without sonication. The incubation took place under a 12:12 h light–dark cycle for one week. The enzymatic activity of SOD was evaluated based on SOD kits which were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and the GSH level was based on a GSH kit supplied by Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China). Chen et al. (2012c) found with increasing Fe_3O_4 NM concentration (400–1,600 mg/L), the GSH content of algae was significantly decreased 96 h post exposure. Moreover, the SOD activity significantly increased (<400 mg/L of Fe_3O_4 NMs), after which there was a significant decrease of SOD activity at 1,600 mg/L of Fe_3O_4 NMs. This decrease in the level of antioxidants results in rapid accumulation of ROS.

In an investigation into oxidative stress induced by copper oxide NMs (CuO NMs) in *Chlamydomonas reinhardtii*, CuO NM suspensions were dispersed using a probe sonicator for 2 min with 40% of amplitude (Melegari et al. 2013). The enzymatic activity of CAT and Glutathione S-transferases (GST) were evaluated by UV–vis spectrophotometry using the molar absorption coefficient of these enzymes. Melegari et al. (2013) found an increase in GST activity compared to the control following exposure to 1,000 mg/L of CuO NMs. The increase in GST concentration can be an indicator for the depletion of GSH in detoxification processes (Lee et al. 2012). Moreover, the CAT activity increased by 139% compared to the control, in response to the concentration of 1 mg/L of CuO NMs, and then decreased following 100 mg/L of CuO NMs and over.

Qian et al. (2016) investigated the effect of 1–100 mg/L of Ag NMs on ROS content and antioxidant levels in two types of freshwater microalgae, *Microcystis aeruginosa* and *Chlorella vulgaris*. Ag NM suspensions were dispersed using bath sonication for 30 min at 25°C. ROS content and CAT activity were measured relative to control according to the instruction of the ROS and CAT activity kits (Beyotime

Biotechnology, Haimen, China).The results showed a significant 1.5-fold increase in CAT activity at 1 mg/L after 96 h exposure compared to control, although the amount of ROS per cell in *C. vulgaris* did not increase significantly. Moreover, Ag NMs were more toxic to the antioxidant systems of *M. aeruginosa* compare to *C. vulgaris*. *C. vulgaris* was able to efficiently detoxify the Ag NM-induced ROS through the induction of antioxidant enzymes (SOD, CAT, and GSH).

Bhuvaneshwari et al. (2017) carried out a number of investigations into the involvement in oxidative stress of Fe NMs on the freshwater green algae *Scenedesmus sp*. up to 72 h post exposure. SOD activity was determined in the supernatant by inhibition of nitroblue tetrazolium (NBT) using a reaction mixture of Na_2CO_3, methionine, NBT, EDTA, riboflavin and phosphate buffer. GSH level was determined based on a Glutathione Reductase Assay Kit (Catalogue Number: GRSA, Sigma-Aldrich) and CAT activity in the supernatant was determined by monitoring the disappearance of H_2O_2, which was done by measuring a decrease in absorbance at 240 nm. The results showed that SOD activity significantly decreased with increasing concentrations of Fe NMs (10, 100 and 1,000 mg/L). In addition, the GSH level significantly decreased with increasing Fe NM concentrations. Moreover, the concentration-dependent increase in CAT activity was noted at all time points (24, 48 and 72 h).

More recent investigations have been carried out on the effects of 0.1 and 1 mg/L of Ag NMs on the antioxidant system of the alga *Chlorella Vulgaris* (Zhang et al. 2017b). The stock suspension of Ag NMs used was 400 mg/L in F/2 medium, with the suspension probe sonicated for 30 min before use. SOD and CAT were measured using a diagnostic reagent kit from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. *Chlorella vulgaris* exposed to Ag NMs for 96 h have shown significant increases in SOD and CAT activity (Zhang et al. 2017b). The authors suggested that the primary mechanism of Ag NMs' toxicity toward *Chlorella Vulgaris* was oxidative stress which evidenced by the breakdown of the antioxidant defence system.

In terms of carbon-based NMs, Thakkar et al. (2016) investigated the effect of 0.1–20 mg/L of SWCNTs on the marine alga *Dunaliella tertiolecta*. NM suspensions were prepared by bath sonication for 30 minutes. Results confirmed a significant decrease (95%) in total GSH level compared to the control when exposed to 20 mg/L of SWCNTs for 120 h. Moreover, the authors concluded that the SWCNTs can cause harm by affecting cellular glutathione levels in *D. tertiolecta*.

Previous investigations into the cytotoxicity effects of MWCNTs on the marine alga *Dunaliella tertiolecta* have shown significant decreases in GSH level with further increases in MWCNT concentration (>5 mg/L) (Wei et al. 2010b). MWCNTs were suspended in Milli-Q water at 1 mg/mL. Thus, *D. tertiolecta* cells experienced substantially higher oxidative stress when exposed to MWCNTs even at low MWCNT concentration (<5 mg/L).

In general, the results of the outlined studies demonstrate that different types of NMs can cause antioxidant response in algal species. There were different approaches used to assess antioxidant response in algal species induced by NMs, such as different types of commercial kits, UV–vis spectrophotometry and inhibition of nitroblue tetrazolium (NBT) using a reaction mixture.

The results of the outlined studies demonstrate that different types of NMs can have an effect on antioxidant levels in algal species. The existing studies did not often look at ROS production and antioxidant levels at the time of the experiments, even though itis an important factor to help identify whether the NMs actually cause oxidative stress. In addition, it is observed that the experimental model (including species used, concentration and exposure method) had an impact on the level of ROS generation as well as the antioxidant defence system in algae, which complicates comparisons between different investigations.

Moreover, DNA damage, lipid peroxidation, membrane damage, oxidative stress and cytotoxicity are also common findings. Additionally, no literature examining the effects of metal-based NMs (in particular Ag NMs) and oxidative stress on *R. subcapitata* at lower concentrations (<1 mg/L) in OECD 201 medium were found.

A summary of some studies on ROS production and antioxidant activity/levels used to evaluate NM-induced oxidative stress using algae species is presented in Table 5.1.

Table 5.1: Reactive oxygen species (ROS) production and antioxidant enzymes used to evaluate NM-induced oxidative stress using algae species

NMs	Concentration (mg/L)	Time (h)	Species	Biomarker	Response	Reference
Ag NMs	1–100	96	*Chlorella vulgaris* and *Microcystis aeruginosa*	CAT	Significant increase by 1.5 × in CAT activity at 1 mg/L after 96 h exposure compared to control. The amount of ROS per cell in *C. vulgaris* did not increase significantly. *C. vulgaris* could efficiently detoxify the Ag NM-induced ROS species via induction of antioxidant enzymes.	(Qian et al. 2016)
Ag NMs	0.01–10	24	*Chlorella vulgaris* and *Dunaliella tertiolecta*	ROS	ROS production induces lipid peroxidation and a decrease of cell viability.	(Oukarroum et al. 2012a)
Ag NMs	0.1 and 1	96	*Chlorella vulgaris*	SOD CAT	Significant increases in SOD and CAT activity. The primary mechanism of Ag NM toxicity toward *Chlorella Vulgaris* is oxidative stress.	(Zhang et al. 2017b)
GO Graphene oxide	0.5–100	96	*R. subcapitata*	ROS	A significant increase in the levels of oxidative stress and membrane damage was observed for concentrations starting at 10 mg/L.	(Nogueira et al. 2015)
SWCNT	0.1–20	120	*Dunaliella tertolecta* (marine)	GSH	Significant decrease in total GSH level compared to the control when exposed to 20 mg/L.	(Thakkar et al. 2016)
MWCNT	0.1–10	96	*Dunaliella tertolecta* (marine)	GSH	GSH decreased with further increase in MWCNT concentration.	(Wei et al. 2010b)
TiO_2 NMs	0.05–1	72	*Scenedesmus obliquus*	ROS	A significant increase in the ROS production was observed in the treated algal cells (1 mg/L) after 24 and 72 h as compared to control	(Dalai et al. 2013)

Fe_3O_4 NMs	50–1600	168	*Chlorella vulgaris*	GSH	With increasing Fe_3O_4 concentration (400–1,600 mg/L), the GSH content was significantly decreased.	(Chen et al. 2012c)
				SOD	The SOD activity significantly increased (<400 mg/L), then there was significant decrease at 1,600 mg/L of Fe_3O_4 NMs.	
Fe NMs	10, 100 and 1,000	72	*Scenedesmus sp.*	ROS	ROS generation was increased by 40% after exposure to Fe NMs for 72 h.	(Bhuvaneshwari et al. 2017)
Zero-valent iron NMs				SOD	SOD activity was significantly decreased with increasing NM concentrations.	
				GSH	GSH activity was significantly decreased with increasing NM concentrations.	
				CAT	The concentration-dependent increase in CAT activity was noted at all time intervals.	
CuO NMs	0.1–1000	72	*Chlamydomonas reinhardtii*	GSH	Increase in activity under CuO NM concentrations above 10 mg/L to 67% compared to control.	(Melegari et al. 2013)
				CAT	Increase of 139% compared to control to the concentration of 1mg/L CuO NM, and then rapid decline of activity.	
Cr_2O_3 NMs	10, 100, 1,000 and 10,000	72	*Chlamydomonas reinhardtii*	ROS	ROS level increased by 38.5%, 63.39%, and 66.81% at Cr_2O_3 NM concentrations of 100, 1,000 and 10,000 mg/L respectively, compared to control.	(Costa et al. 2016)
MWCNTs	10, 20, 30, 40 and 50	96	*Nitzschia frustulum*	ROS	ROS was induced by MWCNTs.	(Jia et al. 2019)
				SOD	SOD activity was significantly induced in *N. frustulum* at all tested concentrations.	

5.1.5 *Aims and objectives*

Oxidative stress induced by NMs has been reported in the literature, and therefore a toxicological study focusing on the generation of ROS and the antioxidant activity/levels related to oxidative stress would broaden the knowledge of NM toxicity, and assess the toxicological effects of NMs as well as the sonication process for NMs (bath or probe) via oxidative stress.

The aim of this chapter is to investigate the effect of Ag NMs (NM300k) and MWCNTs (NM400) on ROS generation (post exposure for 2, 6, 24, 48 and 72 h), and antioxidant defence systems such as SOD and CAT activity and GSH content, in *R. subcapitata* post exposure for 72 h in OECD 201 medium. The influence of the sonication method (i.e. bath vs probe) was investigated. *R. subcapitata* were exposed to sublethal concentrations of the NMs used (EC_{50}, half that concentration and a quarter of that concentration) obtained from acute toxicity studies (Chapter 3). They were then subjected to targeted oxidative stress analysis of ROS generation as well as the enzymatic activity of SOD and CAT, and non-enzymatic levels of GSH. The objectives of this chapter are:

- To assess ROS production by Ag NMs and MWCNTs in the algae *R. subcapitata*.
- To assess the effect of Ag NMs and MWCNTs on SOD activity in *R. subcapitata*.
- To assess the effect of Ag NMs and MWCNTs on CAT activity in *R. subcapitata*.
- To assess the effect of Ag NMs and MWCNTs on GSH level in *R. subcapitata*.
- To evaluate the effect of sonication type for tested NMs on oxidative stress in *R. subcapitata*.
- To evaluate the effect of NMs' physio-chemical properties on oxidative stress in *R. subcapitata*.

Research hypotheses

Based on the literature, it was hypothesised that exposure to different NMs might lead to the overproduction of ROS in algae, which leads to the activation of antioxidant defence mechanisms. These mechanisms can contribute significantly to the cytotoxicity of these NMs towards *R. subcapitata*. The hypotheses can be summarised as follows:

- ROS production will be affected by Ag NMs and MWCNTs in the algae *R. subcapitata*.
- Antioxidant defence systems will be affected by Ag NMs and MWCNTs in the algae *R. subcapitata*.
- Oxidative stress in the algae *R. subcapitata* will be affected by the sonication type of tested NMs (bath or probe).
- Oxidative stress in the algae *R. subcapitata* will be affected by the NMs' physio-chemical properties.

Null hypotheses:

- Oxidative stress will be unaffected by Ag NMs and MWCNTs in the algae *R. subcapitata*.
- Antioxidant defence systems will be unaffected by Ag NMs and MWCNTs in the algae *R. subcapitata*.
- Oxidative stress in the algae *R. subcapitata* will be unaffected by the sonication type of tested NMs (bath or probe).
- Oxidative stress in the algae *R. subcapitata* will be unaffected by the NMs' physio-chemical properties.

5.2 Methodology

5.2.1 *Algae culture preparation*

The initial algal biomass concentration in the starting test cultures in the assessment of ROS production must be sufficiently high – 5×10^6 cells/mL – to perform the DCF assay with *R. subcapitata*. For the antioxidant experiments, the initial algal biomass concentration was the same as acute toxicity studies (5×10^3 - 10^4 cells/mL). The algae culture preparation and exposure experiments for the assays described in this chapter were the same as outlined in sections 3.3.1 and 3.3.2.

5.2.2 *Algal medium*

OECD 201 medium was prepared as previously described in Chapter 2, section 2.2.2.

5.2.3 *NM stock suspension preparation*

Ag NMs (NM300k) and MWCNTS (NM400) were bath and probe sonicated as previously described in Chapter 2, section 2.2.4.

5.2.4 *Selection of NM concentrations*

ROS production and levels/activities of antioxidants in *R. subcapitata* were determined in algal cells exposed to sublethal concentrations of used NMs. The concentrations were IC_{50}, half that concentration, and a quarter of that concentration, obtained from acute toxicity studies (Chapter 3, section no. 3.3. 1). The IC_{50} obtained from acute toxicity studies on *R. subcapitata* were 90 and 193 µg/L for bath- and probe-sonicated Ag NMs, respectively, and 28.5 and 6.83 mg/L for bath- and probe-sonicated MWCNTs, respectively.

5.2.5 *Dichlorofluorescein (DCF) assay*

This is an *in vivo* detection of ROS production using DCFH-DA. ROS production was detected using the probe 2′,7′-Dichlorofluorescin diacetate (H_2DCF-DA) and assessed after 2, 6, 24, 48 and 72 h of exposure to NMs. The assay was carried out based on the method described by Amado et al. (2008). DCFH-DA is a nonpolar dye which is converted into the polar derivative DCFH by cellular esterases following internalisation. DCFH is nonfluorescent but is converted to highly fluorescent DCF when oxidised by intracellular ROS or other peroxides.

H_2DCF-DA obtained from Thermo Fisher Scientific (Catalogue number: D399 – UK) was dissolved in ethanol (1.61 mg of H_2DCF-DA in 8.55 mL ethanol) to obtain a stock concentration of 188.30 mg/L. The stock solution was aliquoted and kept at -20° C in the dark until required. The test samples – OECD 201 medium (blank), algae control (medium and algal cell only) and exposed algae – were added to the wells of a 96-well black plate (250 µL) in triplicate. A volume of 10 µL of H_2DCF-DA was added to each well using a multichannel pipette. The plate was placed in the fluorometer plate reader and a single measurement read, which was used as a background measurement. Readings were taken every 5 min, the plate shaken before measurements were taken, at 23° C to a total of 60 mins, at excitation/emission wavelengths of 488/525 nm using a SpectraMax M5 plate reader.

Total fluorescence production was calculated from a graph by integrating the fluorescence units (FU) along the time of the measurement. Figure 5.1 shows the results of a typical run for fluorescence records over time.

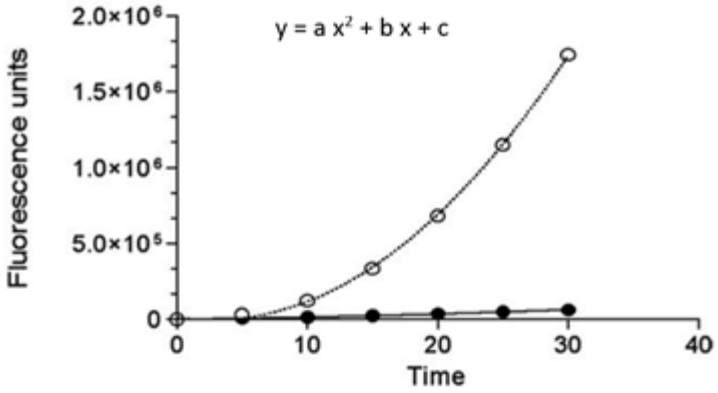


Figure 5.1: Results of a typical run showing fluorescence records over time (Amado et al. 2008)

Then, by calculating the area under the curve through some variable values given from slope curve (a, b & c) (Figure 5.1) for each concentration/time point using the following equation:

$$\text{Area} = (a*60\text{^}3/3)+(b*60\text{^}2/2)+(c*60) \qquad (7)$$

and the level of ROS is expressed as a function of fluorescence units (FU).

5.2.6 *Algae harvesting*

In order to perform antioxidant assays, cells needed to be lysed. Algae cultures exposed to NMs, as well as untreated cultures (as a control), were incubated for 72 h. A volume of 150 mL of each culture in three replicate was extracted at the end of the experiment (72 h). Cells from each treatment were collected by centrifugation (11,200 g for 10 min at 4° C) and re-suspended in 1 mL of 20 mM phosphate buffer (pH 7). Cell disruption was performed on ice with an ultrasonic probe (Lab Sonic) at 10% of power for 5 seconds, followed by a 15-second pause to avoid heat denaturation. This procedure was repeated 6 times. Extracts were centrifuged (11,200 g for 10 min at 4 °C), cell debris were discarded, and supernatant was collected to be used in the antioxidant assays as well as in the Bradford assay for protein content (see Section 3.3.11).

5.2.7 *Superoxide dismutase assay (SOD)*

A superoxide dismutase assay was performed using the appropriate commercial kits, which were purchased from Cayman Chemical (USA) as item no. 706002 (Cayman 2019c). The test algae were cultured for 72 h and then harvested by centrifuging in OECD 201 medium (see section 5.2.6). SOD levels were measured enzymatically, in the clear supernatant based on a tetrazolium salt, for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine.

The assay was conducted according to the manufacturer's instructions (Cayman 2019c). Before starting the SOD assay, several reagents required preparation as per the kit instructions. All reagents were prepared fresh before use. A volume of 3 mL of assay buffer was diluted in 27 mL Milli-Q water in a Falcon tube. A volume of 2 mL of sample buffer was diluted with 18 mL Milli-Q water in a Falcon tube, which was used to prepare the SOD standard and to dilute the xanthine oxidase and SOD samples prior to assaying. A volume of 50 µL of radical detector solution was transferred to another vial and diluted with 19.95 mL of diluted assay buffer, and then the plate was covered with thin foil. For the SOD standard, a volume of 20 µL of the SOD standard was diluted with 1.98 mL of sample buffer to obtain a stock solution. Furthermore, one vial of xanthine oxidase was thawed and a volume of 50 µL of supplied enzyme was transferred to another vial and diluted with 1.95 mL of sample buffer on ice.

At the start of the experiment, a volume of 200 µL of diluted radical detector and 10 µL of the standard were added per well. A volume of 200 µL of diluted radical detector and 10 µL of samples were added to the sample wells. Afterwards, a volume of 20 µL of diluted xanthine oxidase was added to all sample wells. The 96-well plate was shaken carefully for few seconds, then covered and incubated on a shaker for 30 min at room temperature. Finally, a UV absorbance reading was carried out at 440 nm using a SpectraMax M5 plate reader.

According to Cayman (2019c), the SOD activity of the sample was calculated using the following equation:

$$SOD\ (U/mL) = \left[\left(\frac{sample\ LR - y.intercept}{slope}\right) X \frac{0.23\ ml}{0.01\ ml}\right] \qquad (8)$$

The obtained results were divided by the amount of protein for each sample, and data are expressed as SOD activity (U/mg protein).

5.2.8 *Catalase assay (CAT)*

The catalase levels were assessed using a commercial kit, which was supplied by Cayman Chemical (USA) as item no. 707002 (Cayman 2019a). This enzyme catalyses the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water (catalytic activity). CAT levels are measured enzymatically based on the reaction of the enzyme with methanol in the presence of H_2O_2. It is a colorimetric assay (Johansson and Borg 1988; Wheeler et al. 1990). The assay was conducted

according to the manufacturer's instructions (Cayman 2019a). Before starting the CAT assay, several reagents or solutions required preparation as per kit instructions. All reagents were prepared fresh before use. A volume of 2 mL of catalase assay buffer (10x) was diluted into 18 mL Milli-Q water in a Falcon tube to prepare the catalase assay buffer. A volume of 5 mL of catalase sample buffer was diluted into 45 mL Milli-Q water in a Falcon tube and kept in an ice box to prepare the catalase sample buffer. This should be used to dilute the formaldehyde standards, catalase control and CAT sample prior to carrying out the assay. A lyophilised powder of bovine liver CAT was used as a positive control. A volume of 2 mL of diluted sample buffer was added to the vial and vortex, then a volume of 100 µL of the reconstituted enzyme was taken and diluted in 1.9 mL of diluted sample buffer to prepare a positive control. Finally, a volume of 40 µL of catalase hydrogen peroxide was diluted with 9.96 mL of Milli-Q water to prepare catalase hydrogen peroxide.

Furthermore, some CAT reagents were ready to use as supplied, such as catalase formaldehyde standard, catalase potassium hydroxide, catalase purpald and catalase potassium periodate. In terms of formaldehyde standard, a volume of 10 µL of catalase formaldehyde standard was diluted in 9.99 mL of diluted sample buffer.

At the start of the experiment, a volume of 100 µL of diluted assay buffer, 30 µL of methanol and 20 µL of standard were added into formaldehyde standard wells (A–G) in a 96-well black plate. At the positive control wells (+ control), a volume of 100 µL of diluted assay buffer, 30 µL of methanol and 20 µL of diluted catalase were added. At the sample wells, a volume of 100 µL of diluted assay buffer, 30 µL of methanol and 20 µL of sample were added to the wells as planned. After that, a volume of 20 µL of diluted hydrogen peroxide was added to all the wells, then the plate was covered with the plate cover and incubated on a shaker for 20 min at room temperature. Afterwards, 30 µL of potassium hydroxide was added and then 30 µL of catalase purpald was added to each well. Later on, the plate was covered with the plate cover and incubated for 10 min at room temperature on a shaker. Subsequently, a volume of 10 µL of catalase potassium periodate was added to each well, then covered and incubated for 5 min at room temperature on a shaker. Finally, a UV absorbance reading was carried out at 540 nm using a SpectraMax M5 plate reader.

According to Cayman (2019a), the CAT activity of the sample was calculated using the following equation:

$$CAT\ Activity = \frac{\mu M\ of\ sample}{20\ min.}\ X\ Sample\ dilution = nmol/min/mL \qquad (9)$$

The obtained results were divided by the amount of protein for each sample, and data were expressed as a CAT activity (U/mg protein).

5.2.9 *Glutathione assay (GSH)*

The glutathione levels were assessed using a commercial kit, which was purchased from Cayman Chemical (USA) as item no. 703002 (Cayman 2019b). The GSH assay utilizes a carefully optimised enzymatic recycling method, using glutathione reductase, for the quantification of GSH. The assay was conducted according to the manufacturer's instructions (Cayman 2019b).

Before starting the GSH measurement, several reagents required preparation as per the kit instructions. All reagents were prepared fresh before use. A volume of 60 mL of the GSH MES buffer was diluted in 60 mL of HPLC-grade water before use. A volume of 0.5 mL of water was added to the vial of GSH cofactor mixture and mixed well. A volume of 2 mL of diluted MES buffer was added to the vial of GSH enzyme mixture, then capped and mixed well. A volume of 0.5 mL of water was added to the GSH DTNB vial, which was used within 10 min. Furthermore, the GSSG standard was ready to use as supplied. At the start of the experiment, a volume of 100 µL of GSSG standard was added to the wells of a 96-well black plate. After that, a volume of 50 µL of samples was added as planned, then the plate was covered with the plate cover. Afterwards, the cocktail assay was prepared in a 20 mL vial by mixing the MES buffer (11.25 mL), the reconstituted cofactor mixture (0.45 mL), the reconstituted enzyme mixture (2.1 mL), water (2.3 mL) and reconstituted DTNB (0.45 mL). Subsequently, a volume of 150 µL of the freshly prepared assay cocktail was added to each of the wells containing standard and samples using a multichannel pipette, then the plate was covered and incubated in the dark on an orbital shaker for 25 min. Finally, a UV absorbance reading was carried out at 405 nm using a SpectraMax M5 plate reader. According to Cayman (2019b), the total GSH of the sample was calculated using the following equation:

$$Total\ GSH = \left[\frac{(absorbance\ at\ 405\ nm) - (y.intercept)}{Slope}\right] \qquad (10)$$

The obtained results were divided by the amount of protein for each sample, and data are expressed as a GSH level (U/mg protein).

5.2.10 *Statistical Analysis*

All experiments were performed in triplicate on the same day, and the results were presented as means ± standard deviation (SD). Data from all experiments were analysed after checking for normality using the Minitab 18 statistical software package using a general linear model with subsequent ANOVA analysis of variance followed by a Tukey's test. Statistically significant differences were determined using one-way or two-way ANOVA with post hoc multiple comparisons (Tukey) at the level of (p<0.05).

5.3 Results

ROS production and the levels of antioxidant such as SOD, CAT and GSH were assessed in the tested microalgae *R. subcapitata* following 72 h exposure to NMs in order to assess whether oxidative stress was stimulated in algal cells.

5.3.1 *DCF assay*

ROS generation, as assessed by DCF assay in algae cells post exposure to Ag NMs and MWCNTs following bath or probe sonication, is presented in Figures 5.2–5.5. ROS generation was assessed after 2, 6, 24, 48 and 72 h of exposure to NMs. The initial algal biomass concentration in the starting test cultures of the DCF assay was sufficiently high (5×10^6 cells/mL) compared to the initial algal biomass of acute toxicity tests (5×10^4 cells/mL). Exposure of algal culture to NM300k induced an increase in intracellular ROS concentrations.

A time- and concentration-dependent increase in ROS production followed exposure of algae to bath- or probe-sonicated Ag NMs. As shown in Figures 5.2 and 5.3, at 2 h, there was no increase in ROS generation in *R. subcapitata* after exposure to Ag NMs (bath and probe sonicated), compared to the control. However, at 6 h there was a significant increase in ROS generation at the three highest concentrations tested (20, 45 and 90 µg Ag/L and 45, 90 and 193 µg Ag/L of bath- and probe-sonicated Ag NMs NMs, respectively). ROS production by algae exposed to Ag NMs was greatest at 24 h. More specifically, at 24 h exposure to 10, 20, 45 and 90 µg Ag/L of bath-sonicated Ag NMs, ROS generation in *R. subcapitata* was significantly increased by 309.2%, 463.3%, 591.5% and 647.6% respectively, compared to the control sample. In terms of probe sonication, after exposure to 20, 45, 90 and 193 µg Ag/L of NM300k for 24 h, ROS generation in *R. subcapitata* was significantly increased by 383.8%, 618.5%,

710.4% and 826.6% respectively, compared to the control group. At 48 h, there was an increase in ROS production of *R. subcapitata*, but not as much as at 24 h of exposure to bath-sonicated NM300k, and there was no noticeable increase at 72 h.

In terms of sonication type, by using the above-mentioned tested concentrations (20, 45 and 90 µg Ag/L), after 24 h of exposure to Ag NMs suspension prepared by probe sonication a greater response of ROS was induced compared to the suspension prepared by bath sonication. In general, at 24 h, the ROS generation of the algal cells increased with increasing Ag NMs concentration, with significantly higher contents observed at all concentrations of Ag NMs bath and probe sonication (Figures 5.2 and 5.3). In contrast, at 72 h, there was no increase in ROS production, as the risk of nutrient depletion for exponential growth was increased (Figures 5.2 and 5.3).

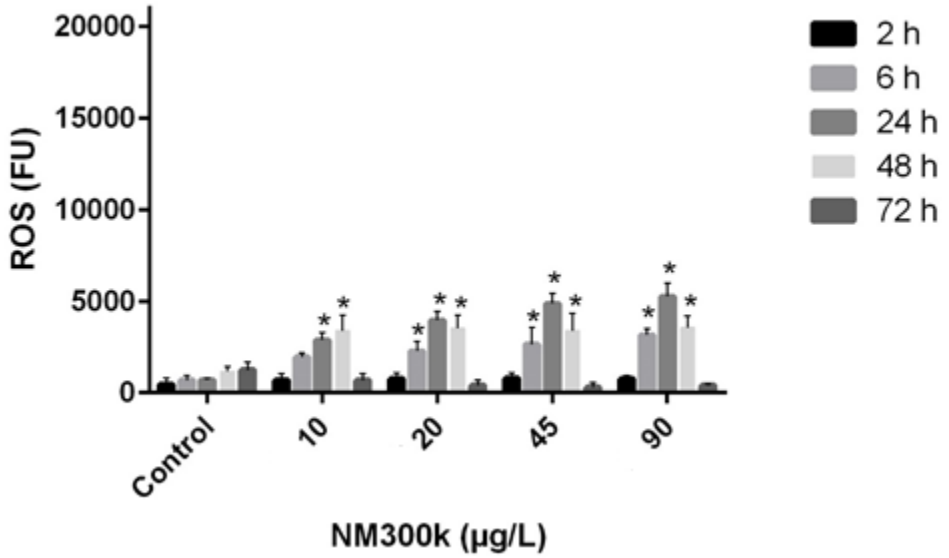


Figure 5.2: Effect of Ag NMs on ROS production in algae. *R. subcapitata* were treated with bath-sonicated Ag NMs for 2, 6, 24, 48 and 72 h. ROS generation was measured using the DCFDA assay. Results are expressed as mean ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

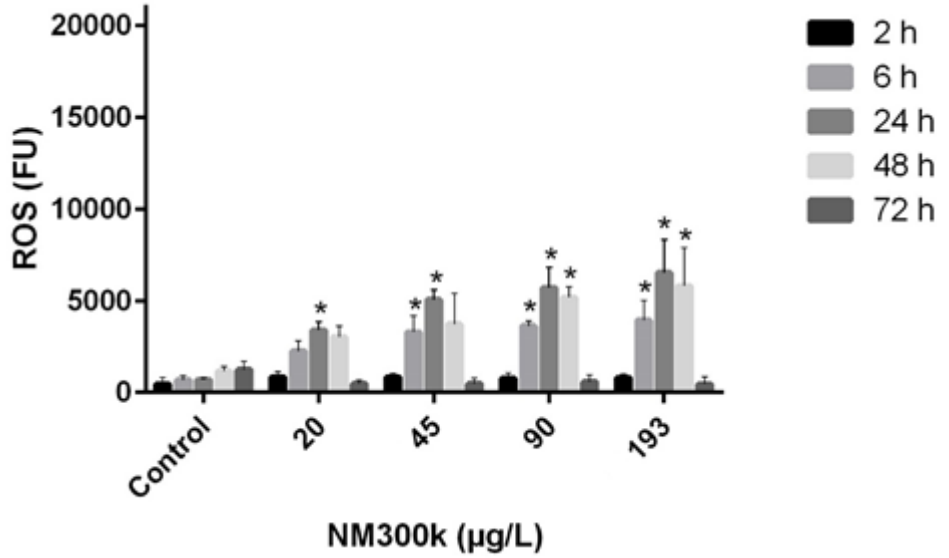


Figure 5.3: Effect of Ag NMs on ROS production in algae. *R. subcapitata* were treated with probe-sonicated Ag NMs for 2, 6, 24, 48 and 72 h. ROS generation was measured using the DCFDA assay. Results are expressed as mean ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

The exposure of algae to MWCNTs induced a time- and concentration-dependent increase in intracellular ROS production. At 2 h, there is no observed change in ROS generation in *R. subcapitata* after exposure to bath-sonicated MWCNTs (Figure 5.4). However, there was a significant increase in ROS generation in *R. subcapitata* after exposure to the two highest concentrations of probe-sonicated MWCNTs (3.5 and 7.15 mg/L of MWCNT) at 2 h (Figure 5.5).

At 6 h there was a significant increase in ROS production at the three highest concentrations tested; 7.15, 14.3 and 28.5 mg/L of bath-sonicated MWCNTs brought increases of 266.4%, 557.9% and 1,394% respectively, and at the two highest concentrations tested of probe-sonicated MWCNTs, 3.5 and 7.15 mg/L, there were increases of 166.3% and 181.4%, respectively.

At 24 h, there was a significant increase in ROS production at the three highest tested concentrations; 7.15, 14.3 and 28.5 mg/L of bath-sonicated MWCNTs saw increases of 453.7%, 1,006.5% and 2,154.3%, respectively. After exposing algae to 1.7, 3.5 and 7.15 mg/L of probe-sonicated MWCNTs for 24 h, ROS levels were increased by 682.7%, 1,404.1% and 2,557.9%, respectively, compared to the control group.

MWCNTs significantly increased ROS levels in *R. subcapitata* at 48 and 72 h post exposure to bath- and probe-sonicated MWCNTs (Figures 5.4 and 5.5). However, the ROS level did not reach the level of ROS production observed in *R. subcapitata* cells at 24 h, as the risk of nutrient depletion for exponential growth was affected (Figures 5.4 and 5.5).

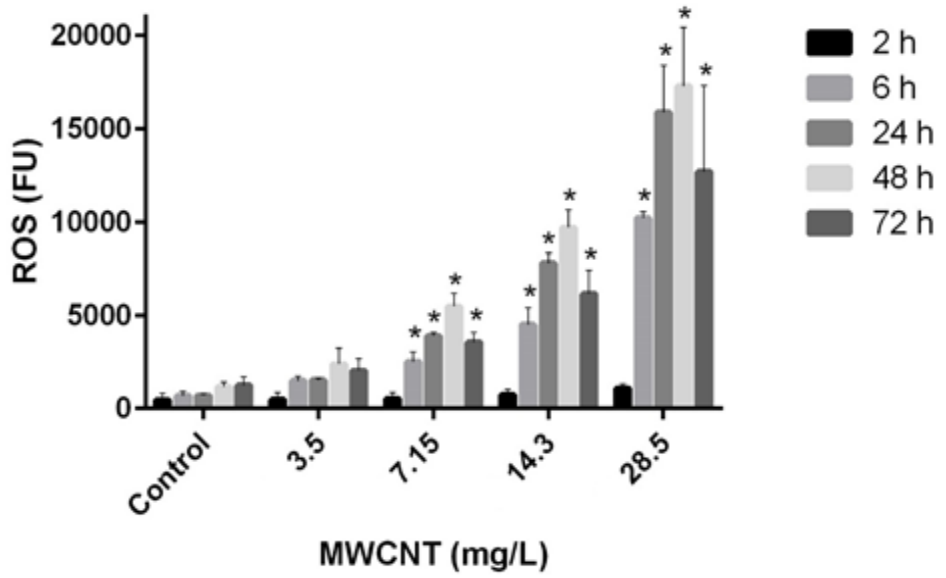


Figure 5.4: Effect of MWCNT on ROS production in algae. *R. subcapitata* were treated with bath-sonicated MWCNTs for 2, 6, 24, 48 and 72 h. ROS generation was measured using the DCFDA assay. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

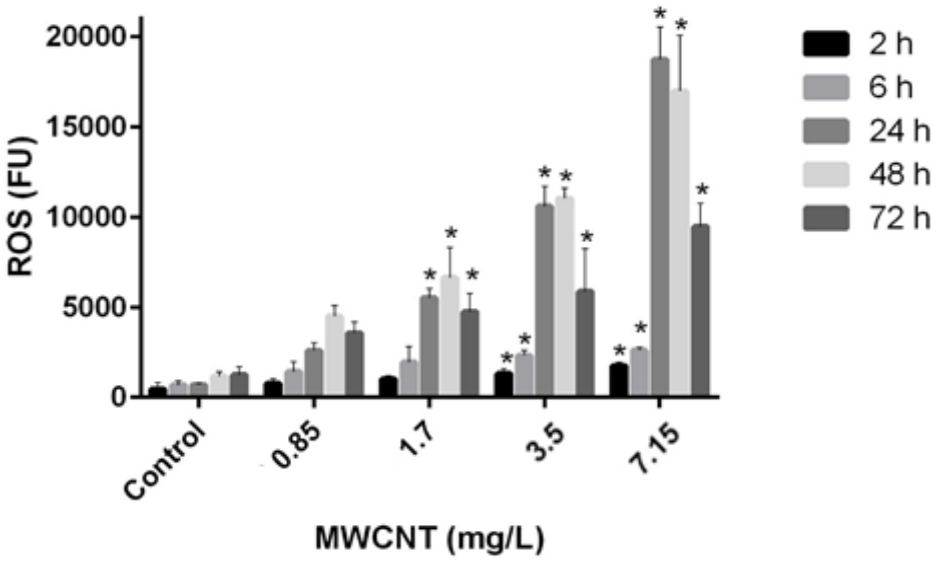


Figure 5.5: Effect of MWCNT on ROS production in algae. *R. subcapitata* were treated with probe-sonicated MWCNTs for 2, 6, 24, 48 and 72 h. ROS generation was measured using the DCFDA assay. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

Generally, at 6 h, the ROS amount in *R. subcapitata* exposed to 20 µg Ag/L of bath- and probe-sonicated Ag NMs was nearly double that of the control sample. Moreover, the ROS level in *R. subcapitata* exposed to 20 µg Ag/L of bath- and probe-sonicated Ag NMs was almost four times higher compared with the control sample at 24 h. However, at 90 µg Ag/L of bath- and probe-sonicated Ag NMs, ROS generation in

R. subcapitata was 6 and 7 times higher compared with the control sample, respectively. This indicates that probe-sonicated Ag NMs stimulated a greater ROS-generating effect in *R. subcapitata* than bath-sonicated Ag NMs.

In terms of MWCNTs, the results obtained after 6 and 24 h of exposure revealed that MWCNTs prompted a significant increase in ROS level. The amount of ROS in *R. subcapitata* exposed to approximately 7 mg/L of bath- and probe-sonicated MWCNTs for 6 h was almost double that of the control sample for both types of sonication. However, after 24 h of exposure to approximately 7 mg/L of bath- and probe-sonicated MWCNTs, the level of ROS had increased by 4 and 25 times compared to the control sample, respectively. Moreover, this also indicates that probe-sonicated MWCNTs stimulated a greater ROS-generating effect in *R. subcapitata* than bath-sonicated MWCNTs. The maximum level of ROS in *R. subcapitata* was noted at the highest tested concentration of 7.15 mg/L of probe-sonicated MWCNT for 24 h, and was 18,726.5 ± 1,784.2 FU.

Based on the results of two-way ANOVA, the interaction of ROS production with sonication type for Ag NMs was not significantly different and the interaction of ROS production with sonication type for MWCNTs was significantly affected by the interaction of the two factors, time points and sonication type (Table

Table 5.2: Results of two-way ANOVA interaction results for ROS production for both type of NMs (Ag NMs and MWCNTs)

NMs	Time	Source	DF	Adj MS	F-Value	*P*-Value
Ag NMs	2 h	Concentration	6	54648	0.71	0.646
Ag NMs	6 h	Concentration	6	2860046	8.21	0.001
Ag NMs	24 h	Concentration	6	8744542	21.97	0.001
Ag NMs	48 h	Concentration	6	4258738	5.53	0.004
Ag NMs	72 h	Concentration	6	299489	3.27	0.032
Ag NMs	72 h	Sonication type	2	877987	11.95	0.284
MWCNTs	2 h	Concentration	3	663995	2.28	0.136
MWCNTs	6 h	Concentration	3	2692795	29.38	0.001
MWCNTs	24 h	Concentration	3	1.2E+08	3.89	0.041
MWCNTs	48 h	Concentration	3	1.06E+08	5.29	0.017
MWCNTs	72 h	Concentration	3	26653151	4.26	0.032
MWCNTs	72 h	Sonication type	2	54734142	16.7	0.001

Furthermore, based on the obtained results, the ROS generated by probe-sonicated MWCNTs was higher than that generated by bath-sonicated MWCNTs, as well as

which, probe-sonicated Ag NMs can generate higher amounts of ROS than bath-sonicated Ag NMs. In other words, the probe-sonicated MWCNTs generate ROS > bath-sonicated MWCNTs > probe-sonicated NM300k > bath-sonicated Ag NMs.

5.3.2 *SOD activity*

To determine whether the antioxidant system was stimulated by Ag NMs and MWCNTs, the superoxide dismutase (SOD) activity was determined. Changes in SOD activity levels in *R. subcapitata* in the presence of NM300k (bath and probe sonicated) are shown in Figures 5.6 and 5.7, respectively. The activities of SOD of *R. subcapitata* were significantly (p<0.05) induced in comparison with the control (Figure 5.6) at all tested concentrations of bath-sonicated Ag NMs; these were 20, 50 and 80 µg/L as (2.05 ± 0.27, 2.43 ± 0.5 and 3.83 ± 0.81 SOD activity U/mg protein) respectively, indicating an oxidative stress of these NMs. However, in terms of probe-sonicated NM300k, the most significant increase compared to the control group was at the highest tested concentration (150 µg Ag/L Ag NMs) (10.23 ± 2.98 SOD activity U/mg protein) (Figure 5.7).

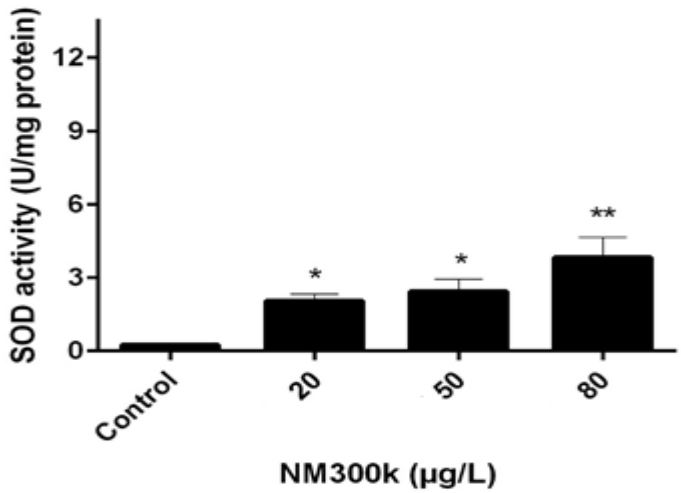


Figure 5.6: Effect of Ag NMs on SOD activity in algae. *R. subcapitata* were treated with bath-sonicated Ag NMs for 72 h and CAT activity was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

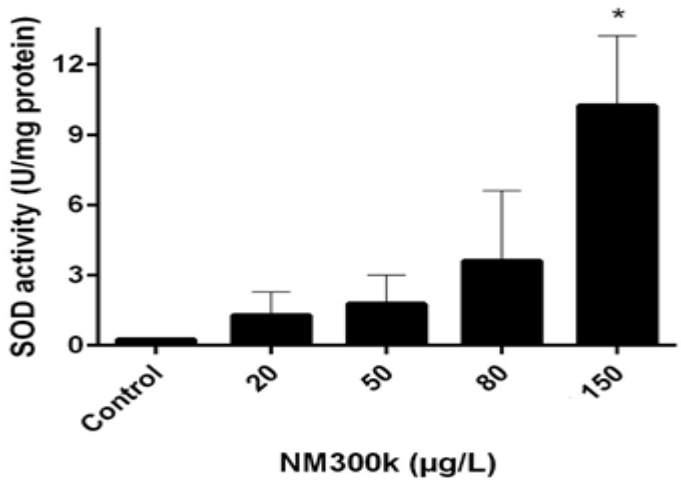


Figure 5.7: Effect of Ag NMs on SOD activity in algae. *R. subcapitata* were treated with probe-sonicated Ag NMs for 72 h and CAT activity was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

In the presence of MWCNTs, SOD activity in *R. subcapitata* increased significantly (p<0.05) with the highest tested concentrations of bath-sonicated MWCNTs, namely 28.5 mg/L (2.71 ± 0.82 SOD activity U/mg protein) in comparison with the control sample (Figure 5.8), indicating an oxidative stress of these NMs. However, in terms of probe-sonicated MWCNTs, the most significant increases were at the two highest tested concentrations (3.5 and 7.15 mg/L of MWCNTs) compared to the control group (3.10 ± 0.66 and 4.01 ± 0.72 SOD activity U/mg protein), respectively (Figure 5.9).

In general, based on the obtained results at 72 h, SOD activity was concentration dependent and significantly stimulated in *R. subcapitata* (p<0.05) by exposure to 20,

50 and 80 µg Ag/L of bath-sonicated NM300k and by 150 µg Ag/L of probe-sonicated NM300k.

SOD activity in *R. subcapitata* was increased by 3.83 ± 0.81 and 3.60 ± 0.83 SOD activity U/mg protein for 80 µg Ag/L of bath-sonicated Ag NMs and probe-sonicated Ag NMs, respectively. However, by increasing the concentration to 150 µg Ag/L of probe-sonicated NM300k, SOD activity was increased to 10.22 ± 2.98 SOD activity U/mg protein. In terms of MWCNTs, at a concentration of 7.15 mg/L of MWCNT, the SOD activity levels in *R. subcapitata* were 0.72 ± 0.24 and 4.00 ± 0.72 SOD activity U/mg protein for bath- and probe-sonicated MWCNTs, respectively. These findings indicate that the probe sonication stimulated greater SOD activity in *R. subcapitata* compared to bath sonication.

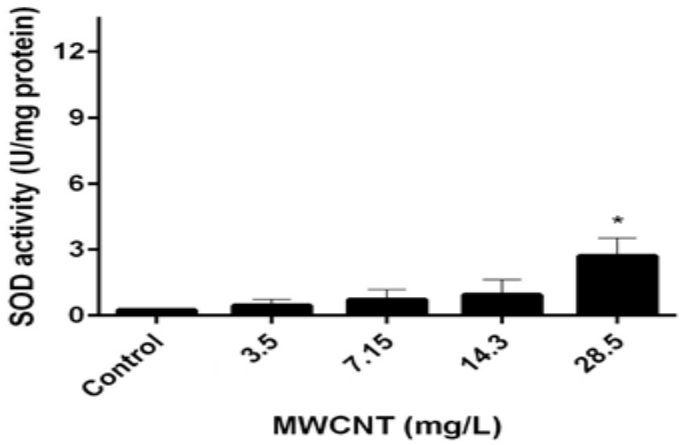


Figure 5.8: Effect of MWCNT on SOD activity in algae. *R. subcapitata* were treated with bath-sonicated MWCNTs for 72 h and CAT activity was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

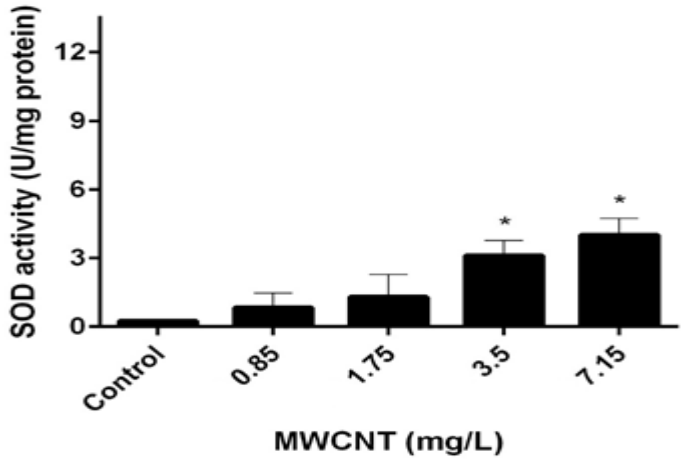


Figure 5.9: Effect of MWCNT on SOD activity in algae. *R. subcapitata* were treated with probe-sonicated MWCNTs for 72 h and CAT activity was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

5.3.3 *CAT activity*

CAT activity was assessed 72 h post exposure of algae to NMs. CAT activity in algae following exposure to bath-sonicated Ag NMs at sublethal concentrations of 20, 50 and 80 µg Ag/L was slightly increased, however, there was no significant difference observed compared to the control (p<0.05) (Figure 5.10). On the other hand, probe-sonicated Ag NMs caused a significant increase in CAT activity (p<0.05) at the highest tested concentration (150 µg Ag/L) (Figure 5.11); CAT activity was 54.87 ± 21.68 U/mg of protein. For MWCNTs, CAT activity in algae exposed to bath-sonicated MWCNTs was significantly increased (p<0.05) at the highest tested concentration (28.5 mg/L); CAT activity was 139.51 ± 44.79 U/mg of protein (Figure 5.12). Similar results were observed for probe-sonicated MWCNTs, as a significant increase (p<0.05) was observed only at the highest concentration tested (7.15 mg/L) and the CAT activity was 133.09 ± 25.48 U/mg of protein. (Figure 5.13).

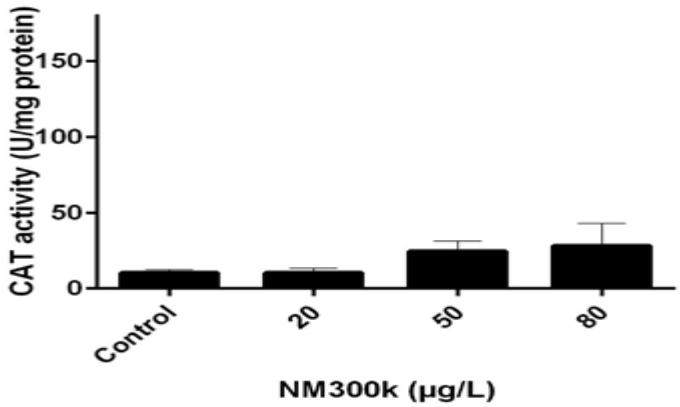


Figure 5.10: Effect of Ag NMs on CAT activity in algae. *R. subcapitata* were treated with bath-sonicated Ag NMs for 72 h and CAT activity was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

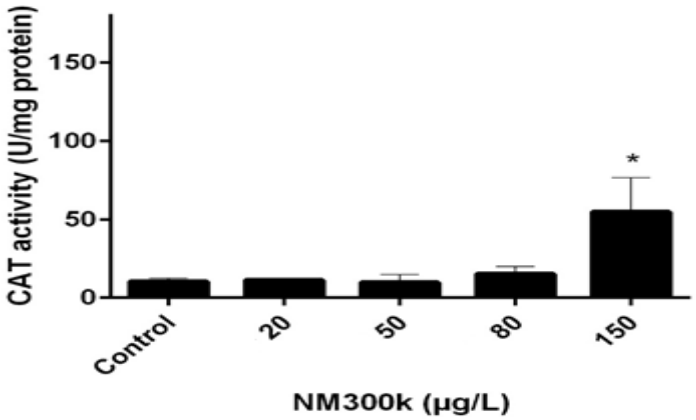


Figure 5.11: Effect of Ag NMs on CAT activity in algae. *R. subcapitata* were treated with probe-sonicated Ag NMs for 72 h and CAT activity was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

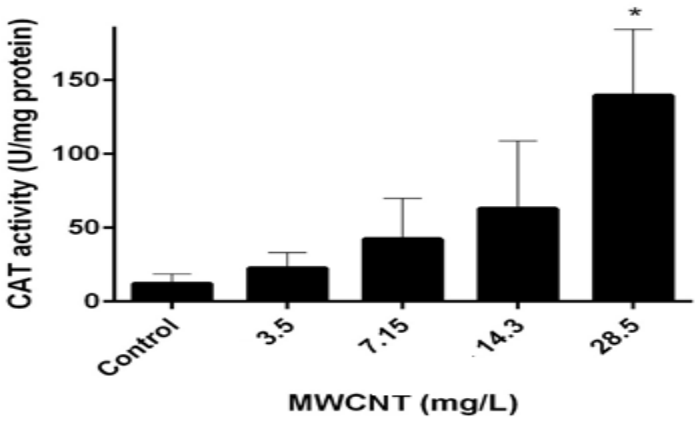


Figure 5.12: Effect of MWCNT on CAT activity in algae. *R. subcapitata* were treated with bath-sonicated MWCNTs for 72 h and CAT activity was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

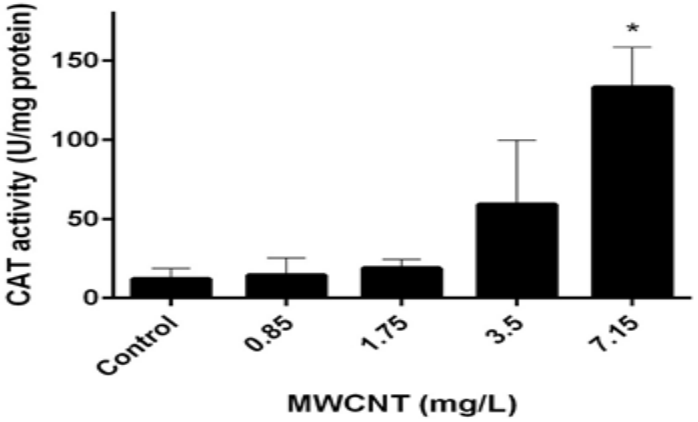


Figure 5.13: Effect of MWCNT on CAT activity in algae. *R. subcapitata* were treated with probe-sonicated MWCNTs for 72 h and CAT activity was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

In general, based on the obtained results at 72 h, CAT activity was concentration dependent. CAT activity in *R. subcapitata* was increased slightly by bath-sonicated Ag NMs and was increased significantly by using probe-sonicated Ag NMs. The amount of CAT activity in *R. subcapitata* reached 54.87 ± 21.68 CAT activity U/mg protein by 72 h post exposure to 150 µg Ag/L probe-sonicated Ag NMs.

In terms of MWCNTs, at a concentration of 7.15 mg/L of MWCNT, CAT activity levels in *R. subcapitata* were 42.17 ± 11.7 and 133.09 ± 25.48 CAT activity U/mg protein for bath- and probe-sonicated MWCNTs, respectively. These findings indicate that probe sonication stimulated a greater effect of CAT activity in *R. subcapitata* compared to bath sonication.

5.3.4 *GSH level*

Glutathione (GSH) levels were determined following exposure of algae to NMs for 72h. At all tested concentrations of bath-sonicated Ag NMs, There was no significant impact on GSH levels (Figure 5.14). However, in terms of probe sonication, the GSH levels were significantly increased at the highest tested concentration (150 µg Ag/L of Ag NMs) reaching 200.95 ± 11.9 U/mg of protein, compared to the control sample (Figure 5.15).

Regarding MWCNTs, all tested concentrations, for both types of sonication (bath and probe), resulted in GSH levels not significantly different from the control, and the main range of GSH levels was between 63.51 and 106.18, and 47.45 and 89.50 U/mg of protein for bath and probe sonication, respectively (Figures 5.16 and 5.17).

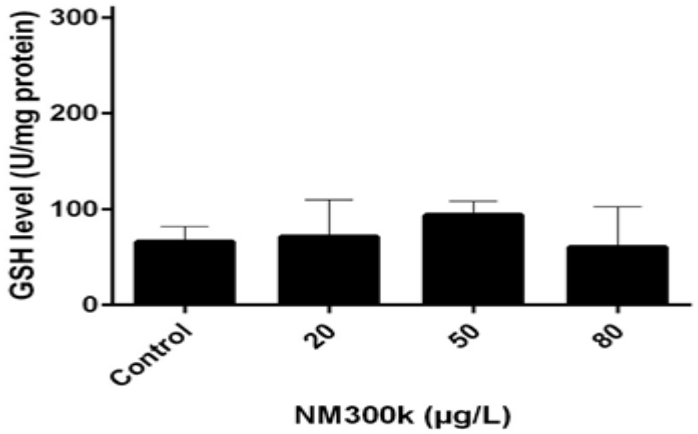


Figure 5.14: Effect of Ag NMs on GSH level in algae. *R. subcapitata* were treated with bath-sonicated Ag NMs for 72 h and GSH level was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

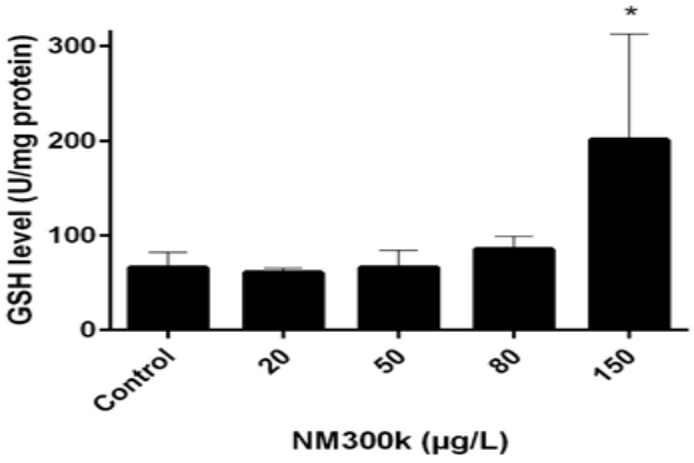


Figure 5.15: Effect of Ag NMs on GSH level in algae. *R. subcapitata* were treated with probe-sonicated Ag NMs for 72 h and GSH level was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

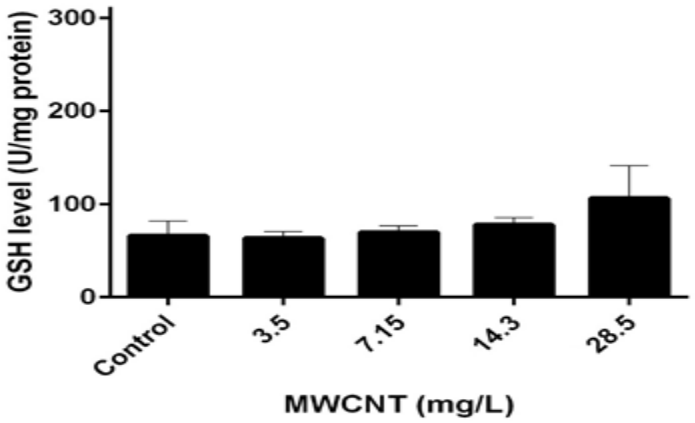


Figure 5.16: Effect of MWCNT on GSH level in algae. *R. subcapitata* were treated with bath-sonicated MWCNTs for 72 h and GSH level was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

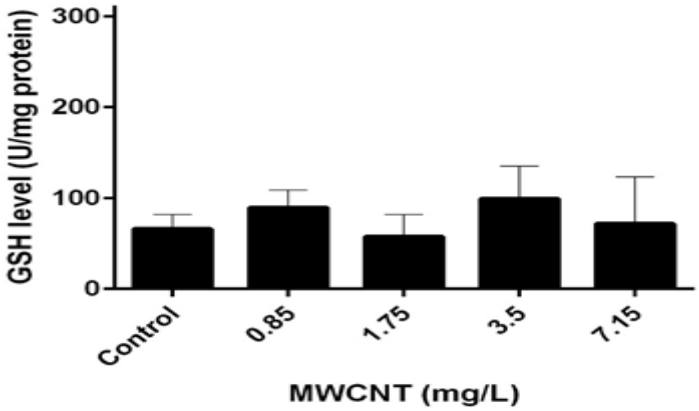


Figure 5.17: Effect of MWCNT on GSH level in algae. *R. subcapitata* were treated with probe-sonicated MWCNTs for 72 h and GSH level was then measured. Results are expressed as means ± SD (n=3). Significance indicated by: *=p<0.05 compared to control.

Based on the obtained results, at 72 h there was no significant increase in GSH level in *R. subcapitata* post exposure to tested NMs, except post exposure to probe-sonicated Ag NMs, where there was significant increase in GSH level at to 150 µg Ag/L of Ag NMs and the amount of GSH level was 200.95 ± 11.9 U/mg of protein. This indicates that probe sonication stimulated a greater effect on GSH levels in *R. subcapitata* compared to bath sonication.

Based on the results of two-way ANOVA, the interaction of SOD and CAT activity with sonication type for Ag NMs and MWCNTs were significantly affected by the interaction of the two factors, time points and sonication type (Table …. and the interaction of GSH level with sonication type for Ag NMs and MWCNTs were significantly affected by sonication type.

Table 5.3: Results of two-way ANOVA interaction results for the antioxidant activity/level for both type of NMs (Ag NMs and MWCNTs)

	NMs	Source	DF	Adj MS	F-Value	*P*-Value
SOD	NM300k	Concentration	6	4.7722	16.45	0.001
SOD	NM300k	Sonication type	2	7.1846	7.06	0.005
SOD	MWCNTs	Concentration	3	6.5362	3.92	0.04
SOD	MWCNTs	Sonication type	2	17.1975	58.21	0.001
CAT	NM300k	Concentration	6	174.84	3.72	0.02
CAT	NM300k	Sonication type	2	234.1	3.4	0.056
CAT	MWCNTs	Concentration	3	5027.5	3.48	0.054
CAT	MWCNTs	Sonication type	2	9363.4	9.17	0.004
GSH	NM300k	Concentration	6	488.6	0.81	0.579
GSH	NM300k	Sonication type	2	105.8	0.17	0.844
GSH	MWCNTs	Concentration	3	834.863	1.07	0.4
GSH	MWCNTs	Sonication type	2	648.8	0.8	0.473

Generally, from the antioxidant studies, SOD activity displayed a similar trend to CAT activity and GSH levels (Figures 5.6–5.17), and all were concentration dependent. Moreover, the finding indicates that the probe-sonicated NMs stimulated a greater effect of antioxidant activity/levels in *R. subcapitata* compared to bath-sonicated NMs.

5.4 Discussion

Investigation of the involvement of oxidative stress in the toxicity of NMs has become a commonly used indicator of toxicity when investigating aquatic toxicity (Shvedova et al. 2012; Zhang et al. 2017b). ROS and antioxidant biomarkers have become central in recent nano-ecotoxicity studies, and such data add value to toxicity testing through clarifying the mode of toxicity and determining the sublethal effects of NMs.

Some studies have reported oxidative stress as one of the mechanisms for toxicity in different types of algae (Kang et al. 2007; Wei et al. 2010b; Schwab et al. 2011; Chen et al. 2012c; Dalai et al. 2013; Melegari et al. 2013; Nogueira et al. 2015; Costa et al. 2016; Li et al. 2016; Qian et al. 2016; Thakkar et al. 2016) (see Section 5.1.1). In the present study, the antioxidants were used as biomarker indicators for the stability of *R. subcapitata* to assess the effect of oxidative stress of NMs on microalgae.

5.4.1 *DCF assay*

ROS plays an important role in maintaining homeostasis under proper conditions at basic levels (D'Autréaux and Toledano 2007), but excessive ROS can lead to

proinflammatory responses such as altering enzymatic activities (Nel et al. 2006; Li et al. 2015a). In this study, the algal cell density in the DCF assay was higher than that in the acute test (the 72 h algal growth inhibition test), since the DCF assay needed sufficient amounts of cellular esterases to detect ROS (Fu et al. 2015). Consequently, the algal cell densities in the two experiments (the ROS generation and the 72 h growth inhibition test) are not comparable. However, the DCF assay gives a great indication of ROS production in *R. subcapitata* after exposure to NMs.

Oxidative stress has been suggested as the primary mechanism of Ag NM toxicity to algae species (Kang et al. 2007; Lyon and Alvarez 2008; Wei et al. 2010b; Schwab et al. 2011; Zhang et al. 2017b). The findings from this study confirm that Ag NMs can generate ROS in *R. subcapitata* using both types of sonication (bath and probe), even at low concentrations (10–190 µg Ag/L) compared to the existing studies in the literature. The concentrations used in this study were lower than those used in the existing literature reviewed using Ag NMs (Oukarroum et al. 2012a; Qian et al. 2016) or using any other type of NMs (Chen et al. 2012c; Dalai et al. 2013; Melegari et al. 2013; Costa et al. 2016; Li et al. 2016; Bhuvaneshwari et al. 2017).

The potential of CNTs to produce ROS in aqueous environments is widely accepted (Pulskamp et al. 2007; Kang et al. 2008; Long et al. 2012). The results demonstrated that MWCNTs using both types of sonication (bath and probe) induced ROS production in *R. subcapitata*. Meanwhile, the results indicated that ROS production was induced by CNTs in *R. subcapitata*, which is in line with the previous studies of NM treatment on aquatic microalgae, including Nogueira et al. (2015) for *R. subcapitata* and Jia et al. (2019) for *Nitzschia frustulum*.

This was a clear indication of oxidative stress, and the absolute value is similar to what was reported before for MWCNTs using different species of algae. Furthermore, the accumulated high amount of intracellular ROS can lead to cell proliferation inhibition, or even cell death via an apoptotic pathway or by necrosis (Kagan et al. 2006; Nel et al. 2006; Zhang et al. 2010b).

ROS levels in *R. subcapitata* at 48 and 72 h post exposure to bath- and probe-sonicated MWCNTs were slightly lower than ROS levels at 24 h, as the cell density in this assay was too high and the risk of nutrient depletion for exponential growth at 48 and 72 h was affected. Moreover, this suggested that the MWCNTs had a shading effect for algae cells and reduced algal inhibition. Furthermore, the MWCNT shading effect has

an impact on this assay, in which some algae cells were hiding behind MWCNT agglomerations, and could produce more ROS compared to NM300k NMs.

The load of impurities common to MWCNTs can contribute to the negative impact of MWCNTs on *R. subcapitata* (Shvedova et al. 2003; Kagan et al. 2006; Smart et al. 2006; Koyama et al. 2009). For MWCNTs used in this study, there were some impurities in the form of metals with the MWCNTs (such as Fe) as detected by EDX analyses. Previous research has linked Fe impurities to toxicity, due to their role in the formation of free radicals and ROS (Shvedova et al. 2003; Nel et al. 2006), and the activity of ROS tends to be related to the concentration of Fe impurities in MWCNT suspensions (Kagan et al. 2006). Moreover, the continuous exposure of algal cultures to light certainly favours different photochemical reactions, such as the Fenton reaction (Youn et al. 2012). With regard to the experimental results obtained in this study, ROS production induced by Fe impurities is possible.

5.4.2 *Antioxidant defence mechanism*

In parallel to assessing ROS production it is essential to measure antioxidant levels/activity to elucidate whether oxidative stress is activated in algae following NM exposure. Torres et al. (2008) indicate that the antioxidant defence systems such as SOD, CAT and GSH can help the organism during oxidative stress. The antioxidant enzymes can be activated by oxidative stress (Yang et al. 2017). An increase in the activity of antioxidant enzymes such as SOD and CAT with increasing NM concentration indicates a clear correlation between free radical generation and antioxidant production, which reflects the oxidative stress caused in the microalgal cells.

However, this activity might be decreased by increasing the toxicant concentration, resulting in a loss of the recovered mechanism (Ramadass et al. 2017). The antioxidant defence system can be inhibited or impaired with a high concentration of NMs as a result of overproduction of ROS, which causes the damage observed to the antioxidant defence system, as evident from suppression of the enzyme activities.

Both NMs were observed to increase CAT activity; MWCNTs induced the greatest effect, probe-sonicated NMs were more toxic than bath-sonicated ones, and the greatest effect was observed at 72 h. This finding was confirmed previously using a higher concentration of Ag NMs (1 mg/L) for 96 h, in two different types of algae – *Chlorella vulgaris* and *Microcystis aeruginosa* (Qian et al. 2016). Furthermore, some

researchers were able to confirm a significant increase in CAT activity using different types of NMs including Fe NMs (Bhuvaneshwari et al. 2017) and CuO NMs (Melegari et al. 2013). Moreover, the concentration-dependent increase in CAT activity was noted at all exposure times (72 h) for Fe NMs in *Scenedesmus sp*. (Bhuvaneshwari et al. 2017).

Meanwhile, the results of SOD activity indicated a significant increase in SOD activity at higher concentrations for both NMs (NM300k and MWCNTs) after 72 h exposure using both type of sonication in *R. subcapitata*. These results are consistent with data obtained in the previous studies of NMs' effects on aquatic microalgae, including Zhang et al. (2017b) for *Chlorella vulgaris*, Chen et al. (2012c) for *Chlorella vulgaris*, Li et al. (2015a) for *Karenia brevis* and Jia et al. (2019) for *Nitzschia frustulum*. Furthermore, Melegari et al. (2013) found a significant increase in SOD activity (139%) at a concentration of 1 mg/L of CuO NMs in *Chlamydomonas reinhardtii* compared to the control sample. When SOD activity is stimulated, its ability to eliminate free radicals is improved at a low concentration of the toxicant, thereby protecting algae from oxidative damage (Sabatini et al. 2011).

This finding is contrary to previous studies which have suggested that there was significant decrease in SOD activity when *Chlorella vulgaris* was exposed to Fe_3O_4 NMs (Chen et al. 2012c) and Fe NMs with *Scenedesmus sp*. (Bhuvaneshwari et al. 2017). This result may be explained by the fact that the authors have used different species of algae as well as different types of NMs.

Regarding GSH levels, there was a slight increase in GSH levels with NM300k, but not significantly compared to the control group. However, this study has been unable to demonstrate if there is a significant effect on GSH levels in *R. subcapitata* post exposure to NMs.

A possible explanation for this might be that a sublethal concentration was used in this research study. Comparison of the findings with those of other studies confirms that there was an increase in GSH level for CuO NM concentrations above 10 mg/L (67%) compared to the control sample (Melegari et al. 2013). This suggests that there is a promotion of antioxidant defences, which is in parallel with findings in this study. In contrast, some researchers found that GSH level was significantly decreased as the concentration increased of different types of NMs such as Fe NMs with *Scenedesmus sp*. (Bhuvaneshwari et al. 2017) and Fe_3O_4 NMs with *Chlorella vulgaris* (Chen et al.

2012c). This suggests that there is an overwhelming effect of oxidative stress defences on GSH levels rather than promotion of antioxidant defences.

Moreover, with MWCNTs, there was no notable trend observed for GSH levels, particularly with probe-sonicated MWCNTs. Wei et al. (2010b) found that the GSH level in *Dunaliella tertolecta* decreased with an increase in MWCNT concentration. Similar results were confirmed by exposing SWCNTs to the same algae *D. tertolecta* (Thakkar et al. 2016).

5.4.3 *General discussion*

In this study, the experiments were performed in an orbital shaker, with a speed of 225 RPM (6 g) and under continuous fluorescent light, based on OECD 201 guidelines for the testing of chemicals. This is necessary to keep the algae in suspension during the test, but is in contrast to previous studies, which were performed in different conditions. The conditions of the present study can allow the algae cells to be in contact with NMs during the experiment period without settling down at the bottom of the test container, and may explain why toxicity was observed at lower concentrations. In addition, the type of algae have to be considered when comparing the sensitivity of NMs among algal species. In addition, the use of different types of algal media and algal culture could have an influence on the degree of the results in the existing studies.

The findings in this research study confirm that there was an upregulation of antioxidant defences, which is the opposite of oxidative stress. This confirms that specimens of *R. subcapitata* were able to protect themselves at the concentrations and time points assessed. This is in line with many other studies. Moreover, the finding shows that MWCNTs were able to generate ROS at a greater level than Ag NMs. However, we have to take into account the NM concentration, as MWCNTs were tested in the range of 1.7–7.15 mg/L for bath and probe sonication, whereas the concentration range of Ag NMs was 10–20 µg/L for bath and probe sonication, respectively. In addition, probe sonication enabled dispersion of NM agglomerates to primary particle sizes, which eases NMs' contact with algal cells and encourages ROS production and the antioxidant. Generally, it was concluded that the probe-sonication dispersion method was a useful way to prepare concentrated and highly dispersed NM suspensions. Furthermore, the change in cell structure and decrease in osmotic

pressure (see Chapter 4) were probably due to damage to the cell wall mediated by ROS production.

5.5 Conclusion

In the present study, sublethal effects of Ag NMs and MWCNTs were studied at the molecular level, using *R. subcapitata*. Findings revealed that the level of stress response varies with the type of NMs and tested time points. The combination of stresses resulted, most likely, in an enhanced pro-oxidant challenge, resulting, therefore, in a different adaptation or compensatory reaction towards ROS formation in order to overcome the stressful condition (Regoli et al. 2011).

Generally, under the experimental conditions used, results indicate that Ag NMs and MWCNTs cause oxidative stress in the microalgae *R. subcapitata*, as evidenced by an increase in ROS production. Moreover, a stronger significant response was produced by antioxidants specifically involved in the stress response of *R. subcapitata* when microalgae are exposed to sublethal concentrations of Ag NMs and MWCNTs. An increase in the activity of antioxidant enzymes such as SOD and CAT with increasing NM concentration indicates a clear correlation between free radical generation and antioxidant production, which reflects the oxidative stress caused in the microalgal cells. In addition, the study highlighted the increase in the induction of the defence system of *R. subcapitata* when subjected to suspension prepared by probe sonication, revealing a significant induction of the antioxidant enzymes compared to suspension prepared by bath sonication.

Furthermore, findings confirm the efficiency of NM300k to produce ROS and enhance the antioxidant defence system in *R. subcapitata* at low levels of concentration compared to MWCNTs. Most of the nanotoxicology studies have focused on identifying and evaluating the effects of large concentrations of NMs on ROS generation and antioxidant enzyme activity in microalgae. However, the present study was focused on the effect of IC_{50} and the sublethal concentrations of NMs, namely half and a quarter of IC_{50}.

Finally, the results highlighted the need for a more holistic determination of antioxidant capacity which would provide a better understanding of a microalga's resistance to toxicity caused by ROS than the measurements of a limited number of antioxidants (Amado et al. 2008), since the antioxidant systems can act in a cooperative way as suggested by the results gathered in this research study.

Chapter 6

General discussion and conclusion

Chapter 6 **General discussion and conclusion**

Due to the distinctive physicochemical properties of NMs, the use of these materials, in a wide range of everyday products (e.g. biomedicine, bioremediation, crop protection, antimicrobials, cosmetics and electronics), has increased exponentially in the last two decades (Tolaymat et al. 2010; Vance et al. 2015; Pu et al. 2016). At present, it is not easy to quantify their release and risk to the environment during their production, use or after disposal (Seager and Linkov 2009; Potter et al. 2019). For example, NMs may undergo a wide range of transformations once in the environment (air, soil and water), such as agglomeration, sorption and functionalisation of other chemicals onto their surface, and dissolution or degradation, which may influence their toxicity (Baalousha et al. 2016).

This research project aimed to assess the toxicity of Ag NMs and MWCNTs to *R. subcapitata*, a model aquatic test organism, over 72 h using the OECD 201 Algal Growth Inhibition Test. In addition, NM interaction with, and internalisation within *R. subcapitata*, and the activation of oxidative stress by these NMs was investigated. The toxicity levels of $AgNO_3$ and Ag NMs DIS (the dispersant within which the Ag NMs are suspended) were also investigated in the same test system, for comparison. The main aims of this research were to evaluate the toxicology of Ag NMs and MWCNTs on aquatic environments, given that these are some of the most widely used NMs to date. Moreover, the influence of two types of sonication – bath and probe – on NM toxicity were compared, since both approaches are widely used to suspend NMs, and little research has been carried out to date to compare how NM dispersion methods might affect their fate and hazard. In parallel to the hazard studies, characterisation of the physico-chemical properties of the NMs was performed in OECD 201 medium (using DLS, and TEM).

Different studies have indicated toxicity of Ag NMs to organisms at almost all biological levels, from cellular level to complete ecosystems, however it is still arguable whether the dissolved Ag, the NMs themselves or a combination of both are responsible for the observed toxicity (Ratte 1999; Wood et al. 1999; Sondi and Salopek-Sondi 2004; Hiriart-Baer et al. 2006; Asharani et al. 2008; Navarro et al. 2008a; Navarro et al. 2008b; Climent 2009; Dror-Ehre et al. 2009; Roh et al. 2009; Meyer et al. 2010; Sotiriou and Pratsinis 2010; Gubbins et al. 2011; Yin et al. 2011;

Levard et al. 2012; Sakamoto et al. 2015; Shen et al. 2015; Li et al. 2017; Abramenko et al. 2018).

In Chapter 2, dispersions of Ag NMs and MWCNTs were prepared in OECD 201 medium by using two different methods: bath and probe sonication. The physico-chemical properties of the NM dispersions were examined and compared. DLS was used for Ag NM characterisation, but this method was not suitable for the characterisation of MWCNTs.

One of the important aims of NM characterisation is to assess the behaviour of NMs in biological media during assays, as well as to link the properties of NMs to their potential toxic effects (López-Serrano et al. 2014). The hydrodynamic diameter and surface charge of the NM dispersions are most likely to be important factors to their hazard potential (Jiang et al. 2009). Therefore, the hydrodynamic diameter and zeta potential of Ag NMs were assessed. Previous studies have demonstrated that Ag NMs form stable suspensions in a variety of media (Hwang et al. 2008; Jung et al. 2008; Klein et al. 2011; Xiu et al. 2011; Wang et al. 2012; Kermanizadeh et al. 2013; Völker et al. 2013; Sorensen and Baun 2014; Gunsolus et al. 2015; Navarro et al. 2015; Donnellan et al. 2016).

Investigations into the effect of sonication method on DLS-derived Ag NMs characteristics (hydrodynamic diameter and zeta potential) showed little difference between the two methods. It was observed that there was a slight increase in the hydrodynamic diameter of Ag NMs when using bath and probe sonication, across all tested concentrations, compared with the original nominal size. It was found that the probe-sonication was more monodispersed than in the bath-sonication to prepare the NM suspensions. An improvement in the agglomeration state of the NMs was observed, as less agglomerated NMs with probe sonication method, which was considered to be the result of the improved efficiency of probe sonication to disperse the NMs agglomeration compared to bath sonication. Furthermore, in terms of zeta potential, the probe-sonication method has a greater effect on the zeta potential of NMs than bath sonication. It was observed that the probe-sonicated Ag NMs had a great negative value for zeta potential, compared with the zeta potential observed for bath-sonicated Ag NMs.

TEM was used to assess the morphology and size of both NMs (Ag NMs and MWCNTs) using both sonication methods. Two methods were used for TEM sample preparation; drop casting and plunge freezing for both NMs (Ag NMs and MWCNTs),

as previously described. Ag NMs had a spherical structure after both bath and probe sonication, and there was no observable effect of the sonication method on the size or morphology of Ag NMs. These findings are in accordance with other published studies which investigated the morphology of Ag NMs in different media using TEM (Klein et al. 2011; Wang et al. 2012; Kermanizadeh et al. 2013; Losasso et al. 2014; Sorensen and Baun 2014; Donnellan et al. 2016; Kleiven et al. 2018). In addition, Ag NMs was confirmed to have a diameter of approximately 20 nm, which showed a good agreement with the manufacturer's specifications and previous studies (Klein et al. 2011; Wang et al. 2012; Kermanizadeh et al. 2013; Völker et al. 2013; Losasso et al. 2014; Mallevre et al. 2014; Johnston et al. 2015; Donnellan et al. 2016; Kleiven et al. 2018).

Regarding MWCNTs, it was found that bath-sonicated MWCNTs formed large agglomerates. In contrast, in the case of probe sonication, it was noticed that there was a lower agglomeration level, especially when using the plunge-freezing process for TEM sample preparation. MWCNTs appeared to consist of entangled, irregular, and bent nanotubes, and it was difficult to measure the dimensions of these nanotubes, which showed a good agreement with the previous studies (Kermanizadeh et al. 2012; Phuyal et al. 2018). Results obtained indicated that the plunge-freezing process is a good procedure for TEM sample preparation compared to the drop-casting procedure. This finding was in agreement with Hondow et al. (2012), since the aqueous phase vitrifies without significant redistribution of suspended NM. In summary, results obtained indicate that probe sonication was more efficient in dispersing MWCNTs.

Previous authors have stated that depending on the duration and type (bath or probe) of sonication, nanotubes might be shortened to different lengths (Huang et al. 2002). Kwok et al. (2010) used two dispersion methods for DWCNTs (ultrasonic bath and continuous stirring) for 2 h and 2 weeks, respectively. Their findings confirm that ultrasonic-bath-sonicated DWCNTs had a significantly smaller agglomerate size than stirred DWCNTs. A shortened MWCNT length reduces the aspect ratio and deteriorates the mechanical and electrical properties of MWCNTs (Park et al. 2012). Indeed probe sonication allows for a relatively efficient preparation of suspended CNTs of a potentially selectable average length (Li et al. 2007; Huang et al. 2002; Meija et al. 2011), although this is not what is aimed when using sonication methods to disperse NMs.

Luo et al. (2010) examined the effect of the ultrasonication process (probe sonication), for 0.5, 2, 4 and 12 h, on the structural changes of bundles of SWCNTs using an ultracentrifuge method and simultaneous Raman scattering and photoluminescence spectroscopy. It was demonstrated that with increased processing time, the cutting-induced length reduction and the diameter decrease of the SWCNT bundles due to nanotube separation were evident, and a continuous decrease of the SWCNT bundle length and diameter was observed in the ultrasonication process. However, in this thesis, the sonication duration time was 2 × 8 min for bath sonication and 14.11 min for probe sonication in ice bath. Moreover, the sonication duration time in this thesis did not reach the lowest tested sonication time (30 min) that have effects on the SWCNT length and diameter in the previous discussed study conducted by Luo and colleagues (2010). Thus, probe sonication is not recommended to disperse CNTs.

Agglomeration of NMs reduces the surface-area-to-volume effects on NM reactivity. In addition, increased agglomerate size can affect NM transport in porous media, sedimentation, interaction with biological systems, uptake by organisms, and toxicity. Over time, agglomeration of NMs into clusters in aqueous medium is inevitable, even if they have engineered or incidental coatings to decrease agglomeration (Lowry et al. 2012). However, the decrease in specific surface area will also depend on particle number, size distribution, charge and the fractal dimensions of the agglomerate (Hotze et al. 2010).

Agglomeration can decrease toxicity when the toxic response of NMs is a result of a surface-area-mediated reaction such as ROS generation or dissolution (Lowry et al. 2012). Agglomeration may also serve to increase the persistence of the NM in the environment if agglomeration decreases the rate of dissolution or degradation (Lowry et al. 2012). The size of a NM in suspension may also affect its bioavailability to organisms. When agglomerates become too large for direct transport across the cell wall or membrane, uptake may be prevented (Lowry et al. 2012).

Following TEM, EDX analyses were used to identify the Ag metal in the TEM samples, as well as the purity of the NMs in general (Brown et al. 2014; Gliga et al. 2014; Klingberg et al. 2015). Surface morphology and the presence of impurities in Ag NMs and MWCNTs were determined by TEM and EDX. The MWCNTs had impurities on their surfaces. Elemental analysis using EDX indicated that MWCNTs contained residual Fe, Al, Cu, Co, S, Na and Si and corundum crystal content

originating from the catalyst used during the synthesis of MWCNTs. These findings are in accordance with Rasmussen et al. (2014).

Visual examination of the NM dispersions was conducted for bath- and probe-sonicated MWCNTs. It was confirmed that the stability of probe-sonicated MWCNTs was greater than that of bath-sonicated MWCNTs in OECD 201 medium as well as in Milli-Q water, as there was more MWCNTs settling down. Finally, results show that probe sonication is more effective in the dispersion of NMs in OECD 201 medium compared to bath sonication.

The aim of Chapter 3 was to assess the acute toxicity of Ag NMs, MWCNTs and $AgNO_3$ to the microalga *R. subcapitata* following the standard OECD 201 test guideline (OECD 2011). In this chapter, algae were exposed to the test materials at different concentrations for 24, 48 and 72 h and the algal response was estimated by using the following four methods: Chl a extraction; OD; protein content, and PSII activity. The impact of the sonication method (bath or probe) used to prepare NM dispersions on the toxicity to algae was evaluated. All four approaches used to assess toxicity indicated that Ag NMs and MWCNTs caused a concentration dependent decrease in algal growth. However, $AgNO_3$ was more toxic to *R. subcapitata* than Ag NMs and MWCNTs. The toxicity of the test substances could be ranked $AgNO_3$> Ag NMs> MWCNTs.

The finding from the Chl a extraction experiment with MWCNTs, indicated that the probe sonicated MWCNTs had a greater detrimental effect on the growth of *R. subcapitata* rather than bath sonicated MWCNTs, based on IC_{50} values. The observed data from the OD experiments also illustrated the effect of the tested materials on growth inhibition of *R. subcapitata*. However, the IC_{50} values obtained from this experiment were higher than the IC_{50} values obtained from Chl a extraction experiments. This finding suggests that there was interference of NMs with OD measurements, which lead to higher IC_{50} values. Moreover, the different IC_{50} values might be related to different methods that vary in their sensitivity. Based on the Bradford assay, the protein content was enhanced over the control values at the lower tested NM concentrations while it decreased with the higher tested concentrations. This finding is in parallel with the findings of Jiang et al. (2014) who found that the protein content of *Spirodela polyrhiza* was inversely correlated with the concentration of Ag.

Photosynthetic activity was also investigated to compare the effects of Ag NMs, $AgNO_3$ and MWCNTs on *R. subcapitata* at 4h and 24h post exposure. Following exposure to all test materials, there was a significant increase in the PSII inhibition of *R. subcapitata* compared to the control samples. These findings are in line with the findings of Navarro et al. (2015) who found that the PSII of *C. reinhardtii* is negatively affected following Ag NM treatment. Moreover, bath-sonicated Ag NMs were more toxic to *R. subcapitata* than probe-sonicated Ag NMs when PSII activity was used as an indicator of toxicity. This finding supports the finding from the Chl a extraction experiment with Ag NMs, which found that bath-sonicated Ag NMs were more toxic than probe-sonicated Ag NMs. In contrast, probe-sonicated MWCNTs were more toxic to *R. subcapitata* than bath-sonicated MWCNTs when photosynthetic activity was used as an indicator of toxicity.

Based on all approaches, the toxicity was enhanced when probe sonication was used, except for Ag NMs. This may suggest that Ag NMs could have stuck on the tip wall of the probe sonicator during the sonication process. This action can lead to the loss of some Ag NM, resulting in the dilution of NM stock suspension (Taurozzi et al. 2011). Another possible reason for this lower toxicity is that Ag NMs might be adsorbed to the walls of the vials during the probe-sonication process. This has been observed in previous studies (Taurozzi et al. 2011). Furthermore, probe sonication might help the NMs to be interact with media compounds such as Cl, S and Si. This interaction with these elements can play an important role in Ag speciation, potentially reacting with Ag and creating complexes that are not available for algae cells (Lee et al. 2005). In general, bath-sonicated Ag NM suspensions affected the growth inhibition much more strongly than probe-sonicated suspensions. The opposite effect of the sonication type on growth inhibition was observed for Ag NO_3 control and MWCNTs.

Interestingly, assessment of NM toxicity to algae is influenced by the endpoint selected to assess algal growth, which can make it challenging to compare findings from different studies. The endpoint most commonly used in studies performed to date were growth rate and growth inhibition. Generally, the Chl a extraction assay is one of the most routinely and reliable tests used to assess NM toxicity to algae. Therefore, where possible, it is recommended that studies should aim to include an algal growth-inhibition test based on Chl extraction when assessing NM toxicity, such as the OECD 201 test. Regarding using OD measurements as an indicator of algal growth, this assay

has certain limitations in terms of interference with NMs as well as using longer time points than other approaches. Furthermore, the lack of standard protocols for other measures of toxicity (e.g. assessment of PSII), coupled with the wide range of potential parameters that can be measured, emphasises the need for harmonisation of approaches (Ralph et al. 2007).

In Chapter 4, the interaction and uptake of the tested NMs (Ag NMs and MWCNTs) with *R. subcapitata* was investigated 72 h post exposure using light and electron microscopy. Based on light microscopy images, there were indications that *R. subcapitata*'s cell morphology was shrunk and damaged, following exposure to both NMs. Greater effects were observed for probe-sonicated NMs, compared to bath-sonicated NMs. Assessment of impacts of NMs on cell morphology has been used previously as an indicator of toxicity. For example, Pakrashi et al. (2013), observed that *Chlorella ellipsoidea* exposed to aluminium oxide NMs (Al_2O_3 NMs) had an effect on cell structure and caused distortions in morphology. NM treated cells showed a reduced size and deformed cell walls. These impacts could be attributed to the destructive effects of ROS (Cabiscol et al. 2000), but a physical interaction between the NM and cell surface and internalisation between cells and NMs could also be important.

There was evidence of Ag NM internalisation by *R. subcapitata* when NM suspensions were dispersed using probe-sonication. However, lower levels of Ag NM uptake were observed for bath sonicated Ag NMs. Most likely, probe-sonicated Ag NMs gave a more dispersed suspension in comparison with bath-sonicated suspensions. For the internalisation of NMs into algal cells, NMs have to move across two barriers on algal cells (i.e. the cell wall and plasma membrane) (Wang and Wang 2014). Navarro et al. (2008b) indicated that the thick and tough cell wall of algae are generally considered the most important barrier to NM internalisation into algal cells. Algal cell walls are semi-permeable, and usually porous (Navarro et al. 2008a). The diameter of these pores is in the range of 5–20 nm (Fleischer et al. 1999; Chen et al. 2016; Zouzelka et al. 2016). Moore (2006), Navarro et al. (2008a), Kalman et al. (2015) and Domingo et al. (2019) have suggested that only NMs that are smaller than the size of the pores (<20 nm) may be internalised across the cell wall. Moreover, the permeability of the cell wall may change during cell division. Kalman et al. (2015) and Xia et al. (2015) indicate that the newly synthesised cell wall is more permeable to NMs, thereby increasing the uptake and internalisation of NMs by algal cells.

Subsequently, NMs can cross the plasma membrane via endocytotic processes (see chapter 4, section 4.4).

Moreover, there is a possibility that suspended Ag NMs interacting with algal cells may lead to an increase in cell wall permeability to facilitate the internalisation of NMs (Ma and Lin 2013). After entering the cells, the NMs are likely to localise in the cytoplasm and organelles, such as vacuoles, and chloroplasts (Ma and Lin 2013). Kalman et al. (2015) investigated the bioaccumulation dynamics of three differently coated Ag NMs and aqueous Ag in a simple freshwater food chain. In their study, Ag NMs were localised in starch granules within the chloroplast of *C. vulgaris*, as confirmed by TEM images. Furthermore, it can be noticed from the TEM images in this study that the white circles in *R. subcapitata* indicate the stress response, showing an increase in the number of starch granules due to NMs exposure. Zhou et al. (2012) observed an increased number of starch granules in *Chlorella pyrenoidosa* after exposure to dissolved zinc and copper. Their findings indicate that the freshwater microalgae *C. pyrenoidosa* developed a defence mechanism against such ionic metals, and this defence mechanism likely isolated the toxicants. Thus, the exposure to NM can increase the number of starch granules in *R. subcapitata* as a defence mechanism against the toxicants.

The consequences of NM internalisation are anticipated to be cytotoxic and oxidative in nature. The size and surface charge of materials has the ability to influence their uptake by cells (Gatoo et al. 2014). In addition, NM size and the cell type under investigation also have the potential to determine the mechanism of uptake and intracellular fate of NMs (Duan and Li 2013).

Ag NMs associated with the cell surface were observed to result in toxicity by pitting the cell walls (Choi et al. 2008; Cherchi et al. 2011; Dalai et al. 2012). The obtained results from the TEM studies confirm that the absorption of bath- and probe-sonicated Ag NMs on *R. subcapitata* cell walls was observed Ag NMs, agglomerates, varying in size, could be visualised externally (Pakrashi et al. 2013; Ribeiro et al. 2015). Moreover, NMs associated with the microalgal surface could inhibit algal growth by agglomeration effects (Schwab et al. 2011). The absorption of NMs on algae might result in a series of subsequent effects from their toxicity, such as physical damage and biochemical injury which might be lead to cell death (Thill et al. 2006; Choi et al. 2008; Wei et al. 2010a; Dalai et al. 2012). Furthermore, the change in cell structure can be potentially related to a decrease in the cellular turgor pressure likely resulting

from the structural wall and membrane damage, mediated by ROS production (Nel et al. 2006; Klaine et al. 2008a; Oukarroum et al. 2012b).

The agglomeration of Ag NMs and MWCNTs would have likely resulted in shading on algal cells, which would have led to reduced light transmittance and reduced viability of algae. For example Schwab et al. (2011) found that shading and agglomeration of CNTs as the main reasons for the toxicity towered algae. These authors indicated that 85% of the total toxicity was caused by the shading effect of CNTs and the agglomeration of microalgal cells with CNTs.

The amount of NMs absorbed by algae cells from aquatic environments can be affected by several factors. One of the most important factors is the acidity (pH) of the environment (Schwab et al. 2016; Wang et al. 2019). A relatively strong pH can enhance the adsorption of NMs to algal cells (Vijayaraghavan et al. 2011). At the same time, the shape and biomass density of algal cells have effects on the absorption of NMs into algae (Esmaeili and Beni 2015).

TEM analysis showed no evidence of internalisation of MWCNTs (bath or probe sonicated) into the algal cells. This could be due to the lack of sufficient contrast to distinguish between carbon-based background cell components and CNTs. However, most algal biomass was trapped in the solid mass of MWCNT agglomerates, with some free algal cells separated from the main cluster. Some free algal cells (from the agglomeration with MWCNTs) appeared to be lost their unique shape compared to control cells. Moreover, the probe-sonicated MWCNTs had a very large effect on cell structure, as confirmed in chapter 4, compared to suspension prepared by bath sonication. It is appears from TEM images that the probe-sonicated MWCNTs were destroyed the intracellular structure of *R. subcapitata*.

In Chapter 5, the role of oxidative stress in NM toxicity to algae was investigated via assessment of ROS generation and the enzymatic activity of the antioxidants SOD and CAT, and levels of GSH. The study confirmed the increase in ROS production of *R. subcapitata* when subjected to NM. In addition, the study highlighted the increase in the induction of the antioxidant system of *R. subcapitata* when subjected to NM suspensions prepared by probe sonication compared to bath sonication. It was found that the activity of the NMs had a significant impact on enzymes of *R. subcapitata* exposed to suspension prepared by probe sonication compared to the suspension prepared by bath sonication. Moreover, these finding comes in parallels with the result obtained from internalisation study (Chapter 4). It was confirmed that the probe

sonicated Ag NMs could be located inside the algal cell and were easier to find in the cells compared to the bath sonicated NMs. Furthermore, with a high amount of NMs inside of algal cells might lead to higher ROS production, leading to damage of algal cells.

Oxidative stress has been suggested as the primary mechanism of Ag NM toxicity to algae (Kang et al. 2007; Lyon and Alvarez 2008; Wei et al. 2010b; Schwab et al. 2011; Zhang et al. 2017b). Moreover, the potential of CNTs to stimulate ROS production in aqueous organisms is widely accepted (Pulskamp et al. 2007; Kang et al. 2008; Long et al. 2012). This study confirms that Ag NMs and MWCNTs can lead to the generation of ROS in *R. subcapitata* using both types of sonication (bath and probe), even at low concentrations (10–190 µg Ag/L) which is much lower concentration compared to other studies in the literature (Qian et al. 2016; Oukarroum et al. 2012a; Zhang et al. 2017b; Wei et al. 2010b; Jia et al. 2019) where a higher concentration were used. The obtained results were in line with other studies of NM treatment on aquatic microalgae, including Nogueira et al. (2015) for *R. subcapitata* and Jia et al. (2019) for *Nitzschia frustulum*. MWCNTs used in this study had some impurities in the form of metals (such as Fe), as detected by EDX analyses. The presence of Fe impurities in MWCNTs has been linked to toxicity due to its role in the formation of free radicals and ROS (Shvedova et al. 2003; Nel et al. 2006). Furthermore, the activity of ROS tends to be proportional to the concentration of Fe impurities in CNT suspension (Kagan et al. 2006). Metal catalyst residues are often very difficult to remove from carbon-based NMs (Lukhele et al. 2015). These metals are often embedded in the tube, and their removal involves using harsh acid treatment which can destroy the nanotube (Lukhele et al. 2015).

Finally, regarding the results presented in Chapter 5, at the same time as assessing ROS production, when assaying the effect of sublethal the doses of NMs it is essential to measure antioxidant levels/activity to elucidate whether oxidative stress is activated in algae following NM exposure, because of the relative activities of the individual antioxidants in the different test systems.

Both NMs were observed to increase CAT and SOD activity; MWCNTs induced the greatest effect, with probe-sonicated NMs being more toxic than bath-sonicated ones, and the greatest effect was observed at 72 h. These results are consistent with data obtained in the previous studies of NM effects on antioxidant activity in aquatic microalgae, including Zhang et al. (2017b) for *Chlorella vulgaris*, Qian et al. (2016)

for *Chlorella vulgaris* and *Microcystis aeruginosa*, Chen et al. (2012c) for *Chlorella vulgaris*, Li et al. (2015a) for *Karenia brevis*, Bhuvaneshwari et al. (2017) for *Scenedesmus sp.* and Jia et al. (2019) for *Nitzschia frustulum*. An increase in the activity of antioxidant enzymes such as SOD and CAT with increasing NM concentration indicates a correlation between free radical generation and antioxidant production, which reflects the oxidative stress caused in the microalgal cells.

In terms of GSH levels, both NMs caused no negative effect on GSH level. Some researchers found that GSH levels were significantly decreased as the concentration increased for different types of NMs such as Fe NMs with *Scenedesmus sp.* (Bhuvaneshwari et al. 2017) and Fe_3O_4 NMs with *Chlorella vulgaris* (Chen et al. 2012c). Generally, findings from this study suggest that there is a promotion of antioxidant defences on GSH levels, rather than overwhelming effect of oxidative stress defence.

Generally, the findings in chapter 2 indicates that the direct method for NMs dispersion (probe sonication) was more effective method compared to the indirect method (bath sonication) for NMs dispersion. That can is in line with the finding from chapters 4 and 5, while probe sonicated NMs appears to be more readily internalise into the algal cell compared to bath sonicated NMs. In addition, the ROS production was more efficient with probe sonication NMs compared to the bath sonication NMs. However, in chapter 3, the findings indicated that the probe sonicated Ag NMs were less toxic than bath sonicated Ag NMs. This might be explained by losing some amount of Ag through sonication processes, specifically with probe sonication. Alternatively, it might be that Ag NMs could have become stuck on vial walls during the probe sonication, somehow resulting in lower availability. Another possible reason for this lower toxicity is that Ag NMs interacted with the probe sonicator tip, which may lead to loss of some amount of Ag NMs in the stock suspension.

In summary, the work presented in this thesis has provided information on the toxicity of Ag NMs and MWCNTs to *R. subcapitata*. It has revealed that the sonication method used to disperse NMs can influence their physico-chemical properties, and also the toxicity of NMs. As well as this, Chl a extraction, OD, protein content and the photosynthetic endpoints showed comparable patterns in Ag NMs and MWCNT toxicity, but differed in their sensitivity. Therefore, the findings could prioritise endpoint selection in future studies such as Chl extraction for their accuracy. Based on the findings from this study it is recommended that activation of oxidative stress is

assessed routinely when investigating the effects of low levels of chemicals/NMs on algae, as the activation of oxidative stress is an important cellular mechanism for toxicity from environmental toxicants, and it is usually resulted from the excessive formation of ROS or the dysfunction of antioxidant defence system (Zhao et al, 2017). The importance of generating reliable characterisation data at concentrations that are toxicologically relevant was highlighted. Therefore, it is recommended to assess the dissolution of NMs in stock suspension at aqueous media before and after sonication process, which can provide beneficial data about losing some amount of NMs during probe sonication, which will be useful at comprehensive characterisation data for NMs as well as in toxicity experiments.

Based on all used approaches, Ag NMs was more toxic than MWCNTs to *R. subcapitata*. This is likely to arise due to differences in particle size, morphology, composition and solubility. In future work it may be valuable to quantify the loss of silver to the test system such as on the probe sonication tip as well as on the NMs stock container. Such losses may be the cause of time dependent reduction in silver toxicity observed (Chapter 3). Moreover, it is strongly recommended to run ICP-MS for some of NMs stock suspension, before and after sonication, to check if there is any loss in NMs concentration. In addition, it is recommended that the response of different species algae is tested and that NMs of more varied physico-chemical properties are investigated, as well as different types of dispersion methods such as magnetic stirring, vortex, and shaking.

Furthermore, there is need for greater environmental relevance in toxicology data, to establish the true threat of predicted increases in products containing NMs in the natural environment, and which ecosystems may be at particular risk. Finally, the mechanism of Ag NM and MWCNT toxicity to algae are still poorly understood, so future studies may be useful to identify the cellular and molecular events underlying any observed toxicity. The conclusions and recommendations made in this thesis are based upon data generated from a limited set of toxicants. As such, future studies should test the extent to which these findings may be applied to other toxicants (including NMs), species and test conditions.

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