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Chapter One - Introduction

1.1. Nanotechnology

The nanotechnology industry has been growing since the 1990s, resulting in a substantial rise in the development of nanotechnology-based consumer products by the mid-2000s (Figure 1.1). Since then, both fundamental research and product development has continued to grow, with an estimated 370,000 total published articles on nanotechnology and 1,800 consumer products released into the market (Figure 1.1) as of the end of 2016 (Project on Emerging Nanotechnologies, 2017). The field of nanotechnology research is highly diverse; at its most complex, it involves manipulation of individual atoms, and has given rise to concepts such as self-assembly, molecular machines and nanomotors. However at present such concepts are mostly theoretical or of limited commercial feasibility, and current nanotechnology research has been applied to developing commercially viable nanomaterials (defined below) for use in consumer products. Engineered nanomaterials (for example silver nanoparticles, carbon nanotubes, and quantum dots) are of varied physico-chemical properties (e.g. size, composition, shape, charge) and are currently being synthesised for use in far-reaching commercial applications, such as textiles (Lo et al., 2007), cosmetics (Mihranyan et al., 2012), medicine (Zhang et al., 2012), and food (Chaudry and Castle, 2011). This industry shows a great deal of potential and a number of governments have dedicated funding for research into nanotechnology, mostly centred around investigating the beneficial effects of nanomaterials and weighing them against their potential harmful effects (i.e. risk assessment). For example, the National Nanotechnology Initiative (USA) and the EU Framework Programme for Research and Innovation (Europe) have funded research to better understand the risks associated with nanomaterials to human health and the environment. With new nanomaterial containing products continually entering the market, research into the potential effects of nanomaterials on both human health and the environment is ongoing. Their unique physiochemical properties drive nanomaterial exploitation; but also make them unpredictable in terms of their potential to cause negative impacts upon both humans and the environment. Indeed, it is established that nanomaterials behave differently to both their ionic and bulk (larger particulate) counterparts, due to their unique physicochemical properties (Handy et al., 2008). Whilst the number of published articles regarding the environmental effects of nanotechnology is approximately 1 % of the total current published research, this proportion has been slowly increasing over the last 5 years (Figure 1.1).
Figure 1.1: Growing research interest in nanomaterials and commercial development of products containing nanotechnology, between the years 1990 and 2016. *Left axis* - Web of knowledge search containing the search terms 'ultrafine OR nanoparticle OR nanoparticulate OR nanomaterial OR nanotube OR nanotubule OR nanofiber OR nanofibre OR nanowire OR nanowisker OR nanorod OR nanoplatelets', organised by year. Papers with a toxicology/environmental science theme are identified as a subset of total research using ‘freshwater OR lake OR river OR aquatic OR sediment OR porewater OR estuarine OR marine OR seawater OR ocean OR terrestrial OR soil’ search terms. *Right axis* - Number of products or product lines containing nanomaterials between the years 2005 and 2016, retrieved from Project on Emerging Nanotechnologies (2017).

The terms nanoparticle and nanomaterial are often used interchangeably, however this is not appropriate. The European Commission has been working on the definition of these materials in recent years, and it is recognised that technically this is a difficult issue that is not yet settled (Bleeker *et al.*, 2013; Rauscher *et al.*, 2017). The current EC working definition of a nanomaterial is:

“A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm- 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.” (EC Directive, 2011/696/EU)
In this thesis the term nanomaterial will be used when generally referring to both nanoparticles and nanomaterials (see definitions below), whereas nanoparticle will be used when referring to specific nanoparticles (for example those used in this thesis, or specific examples in the published literature). The field of nanotechnology has an associated standardised vocabulary, which will be used in this thesis:

- **Nanoparticle**: an object with all three external dimensions in the nanoscale (International Organisation for Standardisation, 2015a).

- **Nanomaterial**: material with any external dimension in the nanoscale, or having internal structure or surface structure in the nanoscale (International Organisation for Standardisation, 2015b).

- **Nanoscale**: size range from approximately 1 to 100 nm (International Organisation for Standardisation, 2015b).

- **Agglomeration**: assemblages of particles held together by relatively weak forces (e.g., van der Waals, capillary, or electrostatic) (International Organisation for Standardisation, 2015a).

- **Aggregation**: discrete assemblages of primary particles that are strongly bonded (i.e., fused, sintered, or metallically bonded) (International Organisation for Standardisation, 2015a).

There are a variety of sources of nanomaterials which can be broadly defined under three main categories; natural, incidental and engineered. Natural nanomaterials include those formed by natural biotic and abiotic processes, such as microbial biomineralisation (Klaus-Joerger et al., 2001) and volcanic activity (Gislason et al., 2011). Incidental nanomaterials are produced as a by-product of human activities, for example diesel exhaust particles which include a range of particle sizes, some of which are in the nanometer range (Sakurai et al., 2003). By contrast, engineered nanomaterials are intentionally produced for a specific purpose (International Organisation for Standardisation, 2015b). Regardless of the source, nanomaterials can form aggregates (chemically bound) or agglomerates (physically bound) that may be considerably larger than the nanoscale (Handy et al., 2008; International Organisation for Standardisation, 2015b). Humans may be exposed to nanomaterials in different settings via a variety of exposure routes (e.g. inhalation, ingestion etc.), including occupational exposure during their production, exposure of consumers during use of products containing nanomaterials, or via the environment. Effects on the environment typically occur
following nanomaterial containing products disposal, natural release (for example from buildings coatings or from cosmetics during swimming), accidental release, intentional release for the purposes of environmental remediation (Khin et al., 2012), and others. Furthermore, during the life cycle of a nanomaterial their physical and chemical properties may be modified by their interaction with other natural substances/processes.

Regulatory bodies require the use of standardised tests to assess the potential hazard of chemicals (including nanomaterials) to both humans and the environment. Indeed, regulations such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals, EU) and the Toxic Substances Control Act (TSCA) of 1976 (USA) require the use of standard tests to assess the toxicity of chemicals. However due to the unique physicochemical properties of nanomaterials, it is not known whether they require separate regulations and testing strategies to their bulk and ionic counterparts, or if standard test protocols designed to assess chemical hazard are suitable. It is important that such concerns are addressed quickly in order to keep pace with existing technological developments, and to ensure that hazard assessment data between laboratories are of sufficient quality to inform robust decision making. Understanding and managing these impacts is crucial, not only from an environmental perspective, but also from a consumer point of view to reduce the chances of reactionary rejection of a promising new technology.

1.1.1. Silver nanomaterials

It has been reported that 15-24 % of all products worldwide contain some form of nanotechnology, with silver the most frequently included element in commercial nanomaterials (Vance et al., 2015; Danish Consumer Council, 2016). Silver nanomaterial containing products include personal care items, cosmetics, clothing, food/food-contact materials, electronics, medicines as well as household and automotive products (Project on Emerging Nanotechnologies, 2017). Whilst the total mass of silver (nanomaterial or ionic) incorporated into such products cannot be inferred from these databases, their inclusion in consumer products is usually based on their well described antimicrobial properties (Nowack et al., 2015; Vance et al., 2015). It is inevitable that silver nanomaterials will be released into the aquatic environment during their production, use and disposal of consumer goods. For example cleaning products and cosmetics containing silver nanomaterials can enter waste water, and textiles containing silver have been shown to quickly leach out their nanomaterials when washed (Benn and Westerhoff, 2008; Geranio et al., 2009). Although some authors debate the novelty of silver nanomaterial toxicity (i.e. beyond merely acting as a source of silver ions) (Emam et al., 2013), there is a considerable need to assess the environmental
impact of increased silver nanomaterial release into the environment, including pathways of toxicity and particle behaviour in the natural environment.

The antimicrobial effects of silver have been known since ancient Greece (ca. 5th century BC), where it was used to preserve food and drink, and to treat wounds (Alexander, 2009). Aside from the rare cosmetic condition of argyrosis or contact hypersensitivity allergies in predisposed individuals, silver contact is of low health risk to humans (Lansdown, 2010). Despite its low toxicity to humans, silver is the second-most toxic metal to aquatic organisms, after mercury (Luoma, 2008). In pristine freshwaters, silver is typically present at low concentrations (ng/l) which makes precise quantification difficult, especially against a background of other contaminants (Luoma, 2008). Whilst the photography industry was considered to be the main historical source of silver pollution of the aquatic environment (Purcell and Peters, 1998), this source is of declining importance due to the rise of digital photography (Mijnendonckx et al., 2013). However the increased production, use and disposal of silver nanomaterials and development of personal care products containing silver in recent years may cause environmental silver concentrations to increase once more (Mijnendonckx et al., 2013). In fact, elevated concentrations of silver have been identified from coastal waters receiving untreated wastewater effluents, and metal mine drainage sites (Tappin et al., 2010).

Silver is not included in the European Commission Directive on Environmental Quality Standards (EQSD) (EC Directive, 2008/105/EC) for the assessment of priority substances in aquatic systems, nor in later proposals for a Directive amending the Water Framework Directive (WFD) and the EQSD (COM(2011)876) which includes a revised (second) list of priority substances. Despite this, silver nanomaterials are one of the 11 nanomaterials listed on the OECD Working Party on Manufactured Nanomaterials (WPMN) priority safety assessment list (OECD, 2010).

1.2. Environmental toxicology and nanomaterials; a challenge to risk assessment

Environmental toxicology is the multidisciplinary approach to studying the effects of toxicants to both terrestrial and aquatic organisms, through controlled laboratory studies. Ecotoxicology is a sub-discipline of this field, which integrates concepts in ecology with toxicology and attempts to gain a better understanding of the effects of substances at the population and community level (Chapman, 2002). Environmental risk assessment seeks to quantify the hazard (toxicity) of chemical substances in natural environments, and combines this information with exposure data to identify the probability/likelihood of adverse effects emerging. By using batteries of test systems (i.e. a group of tests which focus specifically on particular species, endpoints and/or exposure routes), the broader potential impact of a given
chemical can be better predicted with respect to the natural environment (Matzke et al., 2007). Some authors have called for a more holistic approach to environmental toxicology, which utilises *in situ* experimentation and takes into account complex ecological processes such as competition, predation, and community structuring (Chapman, 2002). Whilst such approaches have undeniable environmental relevance, in practice they are difficult to implement from a regulatory perspective (not to mention the ethical consideration of testing chemicals of unknown toxicity in natural environments).

The requirement for environmental risk assessment of new chemicals differs around the world, and is regulated by laws such as the REACH in the European Union, the TSCA in the United States, and Kashinhou (Showa Act No. 177) in Japan. One of the most comprehensive risk assessment guidelines is the internationally recognised Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals, which has developed a set of standard protocols for determining the characterisation and hazards of chemicals on both humans and the wider environment (OECD, 2016). In the EU, REACH requires that specific OECD tests be deployed on the basis of total import/production of a given chemical (EU Directive, 1999/45/EC). These test guidelines must be carefully examined for their suitability to assess nanomaterial safety. For example, the lack of stability of nanomaterials in aquatic media and lack of reproducibility of results between laboratories may be such factors which limit their application. If such tests are found to be unsuitable, a high priority should be placed on the design and validation of new nanomaterial safety assessment protocols, which will inform risk assessments. Such strategies need to be addressed in parallel with the development of nanotechnology as a whole to ensure its safe and responsible development. Accordingly, risk assessment needs to be adaptive to account for the wide range of nanomaterials currently in development, yet rigorous enough to account for nanomaterial-specific differences in both fate and behaviour. In most OECD biotic test systems, concentration response relationships form the foundation of hazard assessment; tests are typically carried out across a range of toxicant concentrations and the change in a specific endpoint (mortality, reproduction, growth, etc.) recorded (OECD, 2004). By using such principles, toxicity can be compared across a wide variety of biotic test systems, using certain common parameters such as effective concentration (EC$_x$, where $x =$ the estimated percentage of endpoint inhibition; usually 50 % of a toxicant free control value), Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) (OECD, 2006a).

At the lowest regulatory threshold of one tonne of an imported/manufactured chemical, REACH requires one invertebrate toxicity test (*Daphnia spp* preferred) and one aquatic plant toxicity test (microalgal species preferred) (EU Directive, 1999/45/EC). For the
latter, the OECD 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test is the most commonly used standard test (European Chemicals Agency, 2016; OECD, 2011). The OECD 201 test was originally developed in 1981, and has been continuously revised to satisfy requirements for rigorous chemical hazard assessment and classification (OECD 1981, 1984, 2006b, 2011). The general principle of the test involves testing the effect of a chemical on the growth rate of exponentially growing cultures of microalgae/cyanobacteria, over 72 hours (OECD, 2011). Experimentally, this is achieved by comparing growth rate (measured as changes in cell counts, or through surrogate measurements of cell growth such as optical density or Chl a fluorescence) in toxicant-free control cultures (six replicates), to that achieved in a series of at least five test concentrations of the chemical to be investigated (three replicates per test concentration) (OECD, 2011). From these data, a concentration which causes a 50% decrease in growth rate (EC50) at a given timepoint (usually 72 hours, but up to 96 hours is permitted) can be derived. Despite its definition as a standard test guideline, there is considerable flexibility in certain test parameters (e.g. test species, medium composition and test duration) (OECD, 2011).

One of the most commonly used test organisms is *Raphidocelis subcapitata* (Figure 1.2). Formerly known as *Pseudokirchneriella subcapitata*, this microalgal species has a sickle shaped appearance and measures approximately 4.8-10.8 µm by 1.6-4.4 µm (Nygaard et al., 1986; Guiry and Guiry, 2016). It has been used in growth inhibition tests to assess the toxicity of metals (De Schamphelaere et al., 2003, 2004; Lee et al. 2004, 2005; Hiriart-Baer et al., 2006; Koukal et al., 2007), herbicides (Chevre et al., 2005; Knauer et al., 2007) antibiotics (Yang et al., 2008; Nie et al., 2013), and nanomaterials (Franklin et al., 2007; Van Hoecke et al., 2008; Aruoja et al., 2009; Hartmann et al., 2010; Ribeiro et al., 2014). Its widespread use in toxicity testing is likely due to the fact that it is fast growing, easy to cultivate, and one of the most sensitive microalgae to a range of toxicants (Rojičková and Maršálek, 1999; Aruoja, 2011). In addition, as algae are primary producers and form the base of most aquatic food chains, any effect on them may have indirect consequences further up the trophic levels of an ecosystem.
1.2.1. Ionic and nanoparticulate silver behaviour in the environment

Ionic silver forms complexes with various ligands in aquatic media. Such ligands include chloride, thiosulphate, and sulphide, as well as naturally occurring organic matter. Compared to seawater, silver speciation in freshwater is far more variable, with free silver (Ag⁺) ions tending to form various silver complexes in solution (Adams and Kramer, 1999; Luoma, 2008). In seawater, speciation occurs with chloride ions (AgCl(s)) making silver in this form much more mobile (Luoma, 2008). By comparison, the formation of silver sulphide (Ag₂S) nanoparticles have been observed in sulphur-rich environments such as that of sewage sludge (Kim et al., 2010). By binding to sulphur, silver can become more insoluble than silver chloride (which can be photo-degraded), and can exhibit greatly reduced toxicity (Nowack, 2010). Therefore silver toxicity may be greatly reduced following release into the aquatic environment and may even provide an effective treatment of wastewater (Blaser et al., 2008). However, some organisms (e.g. algae) may not be protected by this process of sulphidisation due to the presence of membrane-bound sulphate transport proteins, which may result in internalisation of Ag₂S complexes (Fortin and Campbell, 2001; Hiriart-Baer et al., 2006). Little is known of silver that may enter natural waters by other means (i.e. direct run-off of water into natural waters, which bypass waste management facilities), or of the possible further transformation of silver species in natural waters.
The interactions of silver with environmental compounds can have significant effects on both the bioavailability and toxicity of silver to algal cells (Fortin and Campbell, 2001; Hiriart-Baer et al., 2006). Ensuring a constant ionic silver concentration over the duration of a toxicity test is beneficial for establishing reliable concentration response relationships. In practice however, such an aim is difficult to achieve due to silver’s speciation behaviour (described above). Whilst some studies have attempted to ensure constant silver concentrations with ligands, those which are biologically inert (e.g. nitrilotriacetic acid (NTA) and ethylenediamine-tetraacetic acid (EDTA)) have low affinities for silver (Hiriart-Baer et al., 2006). An alternative approach is to use continuous exposures (chemostat) where medium is constantly replenished (Hiriart-Baer et al., 2006). Whilst silver speciation can strongly affect bioavailability, uptake is typically rapid, especially in marine phytoplankton and marine invertebrates (Luoma, 2008).

Silver nanomaterial behaviour in aqueous media depends on particle physico-chemical properties such as size, shape, surface properties, and media composition (El Badawy et al., 2010; Elzey and Grassian, 2010; Kennedy et al., 2012). Excluding very low pH values, most silver nanoparticles tend to dissolve very slowly (Kittler et al., 2010; Ma et al., 2012). Nanomaterials can be manufactured ‘bare’, or can be surface-modified to enhance stability in suspension to prevent agglomeration/aggregation (El Badawy et al., 2010, 2011; Li and Lenhart, 2012). In addition, nanomaterials can be prepared with dispersants (non-ionic stabilising agents), which enhance stability through steric processes (i.e. dispersants which are not irreversibly bound to the nanomaterials; see Chapter 2.1.1 for more information). Non-ionically stabilised silver nanomaterials are highly effective at inhibiting bacterial activity (Li and Lenhart, 2012; Losasso et al., 2014). The properties conferred by surface modification or non-ionic dispersion may cause silver nanomaterials to persist in the environment for longer periods of time, and thereby impact on their toxicity (Fabrega et al., 2011).

1.3. Sources of nanomaterials and the requirements for characterisation

There are several sources of nanomaterials which can be used when investigating their toxicity in the laboratory. One approach to investigating nanomaterial toxicity is to use particles which have been synthesised “in-house” – that is within the institution or research partnership contributing to the study in question. A variety of methods can be used to synthesise these nanomaterials, depending on the composition and desired properties (See Ramazani et al., 2016 for review). Whilst in house synthesis has a number of practical advantages, such as control over all stages of production and the ability to produce particles on an ad hoc basis, slight differences in methodology may confound attempts to compare
findings to other published work (Totaro et al., 2016). Furthermore, such practices may not produce nanomaterials that are representative of the types found in existing consumer products.

A second approach is to use nanomaterials which have been manufactured by commercial suppliers (i.e. industry) for use in products. This allows not only for a high degree of industrial-relevance, but also minimises inter-laboratory variation in studies using the same supplied nanomaterial. Issues associated with this approach, however, are the batch to batch variability (e.g. Crist et al., 2013) and the unquantified amounts of contaminants (often found in such industrial nanomaterials (e.g. Pulskamp et al., 2007)). To address this, the European Union Joint Research Centre (JRC) Nanomaterials Repository was established in 2009 to provide a variety of research programmes with industrially prepared, commercially available nanomaterials. Since 2009, it has grown into a highly esteemed facility, which uses state-of-the-art equipment and good laboratory practices to subsample large industrial batches of nanomaterials with a high degree of precision (Totaro et al., 2016).

In an attempt to address issues of comparisons between laboratories, benchmark materials can be used to facilitate inter-laboratory standardisation (Roebben et al., 2013). These benchmark materials can be defined under one of three categories, depending on their level quality assurance (e.g. homogeneity and stability of certain defined properties):

- **Reference material**: material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process (International Organisation for Standardisation, 2016)
- **Certified reference material**: reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability (International Organisation for Standardisation, 2016)

The concept of certified reference materials is not novel, and such definitions have been refined over many decades (Cali, 1979). Certified reference materials are typically used to calibrate laboratory equipment, and to verify methods used in different laboratories to ensure comparability (Roebben et al., 2013). Examples of non-nanomaterial certified reference materials used in this thesis include Standard Reference Materials 1640a (Trace elements in Natural Water; for the calibration of ICP-MS instrumentation; Chapter 2) and Diuron.
(Photosynthetically active herbicide; for comparative toxicity testing; Chapters 4 and 5). When producing reference nanomaterials, a number of challenges have been identified particularly concerning the most relevant properties to include, and which approaches and test guidelines are most suited to characterise those properties (Roebben et al., 2013). As such few reference nanomaterials exist, and even fewer certified reference nanomaterials, outside of those concerned primarily with particle size (Linsinger et al. 2011; National Institute for Standardisation, 2011; Roebben et al., 2011; Stefaniak et al., 2012). This had led to the defining of a third class of benchmark material:

- **Representative test material**: material, which is sufficiently homogenous and stable with respect to one or more specified properties, and is implicitly assumed to be fit for its intended use in the development of measurement and test methods that target properties other than those for which homogeneity and stability have been demonstrated (International Organisation for Standardisation, 2013)

Under the representative test material concept, if a nanomaterial is demonstrated to be homogeneous and stable for one measured parameter, it is assumed that such stability applies to other properties as well (Roebben et al., 2013). For nanomaterials provided by the JRC, such stability assumptions are primarily obtained from size measurements or particle numbers (as a proxy for dissolution). Examples of such representative test nanomaterials include zinc oxide (NM110 and NM111) (Singh et al., 2011) and silver (NM300 and NM300K) (Klein et al., 2011). It is worth noting that the JRC recently updated the nomenclature of all of its representative NM series (JRC Nanomaterials repository, 2016), but for the purposes of comparison with existing published literature predating the nomenclature change, the previous nomenclature will be used throughout this thesis. Whilst the representative test material concept allows for assumptions of stability to be made upon few characterisation measurements, it does not preclude the need for characterisation of representative test materials in individual studies, which may utilise different media to suspend nanomaterials, temperatures, exposure regimes, etc. Some representative test nanomaterials have been extensively characterised in a range of test media to verify that the information provided by the suppliers was accurate (Klein et al., 2011; Singh et al., 2011). However characterisation of the properties of these nanomaterials in more diverse test media is required. The characteristics of various laboratory synthesised nanomaterials (i.e. non-representative) have been shown to change over time (Phenrat et al., 2008; Kittler et al., 2011; Kuchibhatla et al., 2012) or when suspended in different media (Fabrega et al., 2011; Izak-Nau et al., 2013). This further evidences the benefits of representative materials, which although may still change
over time and in different media, they tend to be more stable, and have been subject to detailed characterisation studies that can be used as guidance. Indeed, characterisation data can be used as a basis for future comparison for hazard studies by those seeking to compare their results to other studies.

One of the key research questions to address in the study of silver nanomaterial effects on the environment, is the extent to which their mechanism of toxicity is novel (i.e. particle specific), as opposed to acting merely as a source of silver ions (Levard et al., 2012). Some studies have suggested an additional toxic effect of silver nanomaterials than that which would be predicted solely on the basis of dissolved ions (Navarro et al., 2008; Fabrega et al., 2009; Sorensen and Baun, 2015). However such studies have failed to identify the cause of such observations, which may include speciation as a result of different test medium composition (Xiu et al., 2012). More compellingly, other studies have attributed the effect of silver nanomaterial toxicity solely to dissolved ions (Hwang et al., 2008; Jung et al., 2008; Xiu et al., 2011, 2012; Gunsolus et al., 2015; Navarro et al., 2015). A number of these studies have ascribed certain environmental parameters that may modify ionic silver release from nanomaterials, and so affect toxicity. In addition, the functionalization (modification/capping) of the nanomaterial surface may influence their toxicity. For example, Xiu et al. (2012) found that polyethylene glycol (PEG) coated silver nanoparticles to which Escherichia coli were exposed under anaerobic conditions, had no lethal effect at concentrations around 7,000 times higher than the minimum lethal concentration of ionic silver, using the same test system. The requirement for dissolved oxygen to facilitate silver nanoparticle dissolution has also been supported by Liu and Hurt (2010), who found that citrate stabilised silver nanoparticle dissolution required both protons and dissolved oxygen, and is governed by the equation (Liu and Hurt, 2010):

\[
2\text{Ag}(s) + \frac{1}{2}\text{O}_2(\text{aq}) + 2\text{H}^+ (\text{aq}) \leftrightarrow 2\text{Ag}^+(\text{aq}) + \text{H}_2\text{O}(l)
\]

Liu and Hurt (2010) also found that natural organic matter (NOM) decreased dissolved silver released from nanomaterials in a concentration dependent manner. The effect of NOM on silver nanomaterial dissolution, and resulting toxicity, is poorly understood. This lack of understanding is likely due to the heterogeneity of both NOM and silver nanomaterials, in addition to differences in methodology used between studies. Results from such research have indicated both an increase (Gondikas et al., 2012; Pokhrel et al., 2013, 2014; Gunsolus et al., 2015) and decrease (Liu et al., 2010; Liu and Hurt, 2011; Wirth et al., 2012) in release of silver ions from silver nanomaterial exposures, in the presence of NOM. In terms of toxicity, most authors agree that NOM reduces toxicity but disagree over the primary mechanism, such as
ionic silver-NOM complexation (Wirth et al., 2012; Zhang et al., 2012) or the adsorption of NOM to nanomaterial surfaces preventing oxidative dissolution (Fabrega et al., 2009; Yang et al., 2014). In addition different forms of NOM may express different affinities for silver, which has both experimental and environmental implications (Gunsolus et al., 2015).

1.4. Alternative testing strategies

Currently, engineered nanomaterials are typically constructed of simple chemical elements (e.g. metals, carbon) or compounds (e.g. metal oxides, polystyrene), but future generations of nanomaterials are expected to include complex structures (Browne and Feringa, 2006). Depending on the specific physical properties of current nanomaterials, it may not be suitable to test their toxicity in existing standard testing systems. Such problems with testing nanomaterials include ensuring constant exposure concentrations (highly aggregating/agglomerating nanomaterials), appropriate dosing strategies, and the influence of test parameters (light, temperature, media composition, etc.) on observed nanomaterial toxicity (Handy et al., 2008). Furthermore, nanomaterials have been recorded as interfering with a range of short term mammalian cytotoxicity tests (Worle-Knirsch et al., 2006; Holder et al., 2012; Xia et al., 2013), automated cell counters (Hartmann et al., 2013) and polymerase chain reaction assays (Wan et al., 2009). As such, there is considerable need for simple but reliable test protocols which avoid potential artefacts of nanomaterial interference. When performing algal testing, nanomaterial physical properties may introduce confounding toxic effects through media/organism interactions (Aruoja et al., 2009; Long et al., 2012), or may change algal growth through indirect toxic effects, such as the shading of light or inducing aggregation of individual cells (Aruoja et al., 2009; Schwab et al., 2011). Furthermore algae may reduce the effect of some toxicants through the production of exopolymeric substances which may chelate metals or generally prevent toxicant-cellular interaction through poorly understood mechanisms (Miao et al., 2009; Zhang et al., 2012).

Since the implementation of REACH in 2007, there has been a considerable shift in focus in the field of regulatory toxicology. This new direction seeks to move away from whole animal vertebrate models, to a tiered screening approach using a selection of in vitro models, with vertebrate models only used for substances of particular concern (Hartung, 2009; European Chemicals Agency, 2014). In addition to addressing the obvious ethical concerns of traditional regulatory toxicology strategies, the new tiered approach seeks to increase test efficiency and reduce time and cost of testing through the use of alternative testing strategies (Hartung, 2009). For algal testing, suggested alternative tests include shorter term exposures (Blaise et al., 1991; van der Heever and Grobbelaar, 1998; Franklin et al., 2005; Navarro et al.,
reduced exposure volume (Arensberg, 1995; Geis et al., 2000; Eisentraeger et al. 2003; Lee and An, 2013), and lower sample handling time/increased automation (Eisentraeger et al., 2003; Hunde-Rinke and Simon, 2006; Lee and An, 2013; Sekine et al., 2015). Whilst some of these proposed alternative testing strategies have been designed using the same growth inhibition endpoint as the standard OECD 201 test (Blaise et al., 1986; Arenseberg et al., 1995; Geiss et al., 2000; Eisentraeger et al. 2003; Franklin et al., 2005; Hunde-Rinke and Simon, 2006; Lee and An, 2013; Sekine et al., 2015; Sorensen and Baun, 2015), others have used novel endpoints to determine substance toxicity as described below.

A number of studies have focussed on developing viability assays, more commonly used within mammalian toxicology (Stone et al., 2016) to algae. Such assays generally focus on metabolic/enzymatic activity (Franklin et al., 2001 Regel et al., 2002; Brickley et al., 2012; Gorokhova et al., 2012; He et al., 2012; Machado and Soares, 2015) or cellular membrane integrity (Sato et al. 2004; Ribalet et al. 2007; Timmermans et al. 2007; Segovia and Berges 2009; Chang et al. 2011; Peperzak and Brussaard, 2011; Machado and Soares, 2015). However, developing stain-based assays for algae may be confounded by physiological differences in dye uptake between species, and difficulties in developing effective positive controls (Gorokhova et al., 2012). An alternative to stain-based approaches of determining toxicity have also been proposed, which focus on photosynthetic activity. Through a transfer of knowledge between plant science and environmental toxicology, algal photosynthetic activity can be determined using a number of methods. These include radiolabelled carbon uptake (Trampe et al., 2015; Soresen and Baum), oxygen evolution (Brindley et al., 2010; Trampe et al., 2015) or through and accessory pigment fluorescence (see Ralph et al., 2007 for review). Fluorometric methods of determining photosynthetic activity are advantageous as they are rapid, non-destructive, and protocols are valid across all photosynthetic taxa (Ralph et al., 2007).

1.5. General aims and objectives

The work described in this thesis aimed to optimise the methodology used to assess the toxicity of nanomaterials to algae, using the OECD 201 test as the foundation for study. In order to study silver nanomaterial toxicity in this work, a representative nanomaterial was used; NM300K as provided by the JRC. These silver nanoparticles are dispersed in a non-ionic surfactant in order to remain well dispersed in a variety of natural and synthetic media and to quickly establish equilibrium in terms of released silver ions (Klein et al., 2011). As a representative nanomaterial, NM300K has been well characterised and shown to be stable across a number of parameters by both the JRC (Klein et al., 2011) and subsequent toxicology
studies (Wang et al., 2012; Kermanizadeh et al., 2013; Volker et al., 2013; Sorensen and Baum, 2015). In order to verify such stability of NM300K in the test systems used in this research, particle size, surface charge and dissolution were studied in two types of algal growth medium, presented in Chapter 2.

It is uncertain whether existing regulatory testing strategies can be deployed to adequately assess the environmental toxicity of silver nanoparticles. In order to address this, first the OECD 201 algal growth inhibition test was used to investigate the toxicity of silver (ionic and NM300K) to \textit{R. subcapitata}. Flexibility within the OECD 201 test protocol, which allows the use of alternative media, may confound comparisons of nanotoxicology experiments conducted between laboratories. For these experiments, test designs included the standard OECD algal growth medium and a non-standard algal culture medium used in routine laboratory husbandry. In addition, studies investigating the effect of humic substances and pH on silver toxicity to \textit{R. subcapitata} were carried out in both media, to partially address concerns over environmental relevancy of standard tests. From a broader environmental perspective, standard test guidelines may not give a sufficient understanding of behaviour/toxicity of nanomaterials in natural systems. These topics were investigated, and results presented in Chapter 3, including results from the investigation of the influence of humic acid and pH on silver nanoparticle toxicity.

Given the increasing number of products containing nanomaterials, there is considerable regulatory demand for adequate and rapid risk assessment. The current OECD 201 guideline suggests flasks should be used as the primary vessels in which to culture algae in growth inhibition experiments. Despite this, a number of authors have had success assessing toxicity of various substances to algae using miniaturised test systems which seek to reduce test volumes and sample processing times (Arenberg et al., 1995; Geis et al., 2000; Okamura et al. 2002; Eisentraeger et al., 2003). In Chapter 4, results from a simple, miniaturised OECD 201 test system were presented. The effectiveness of such an approach was evaluated in determining silver (both ionic and NM300K) toxicity to \textit{R. subcapitata} in OECD medium. In addition, the humic acid and pH experimental approaches presented in Chapter 3 were performed in this miniaturised system in order to test their suitability in gathering more environmentally relevant toxicity data.

Finally, and in keeping with the requirement for more rapid testing strategies, Chapter 5 presented the results from a short term (<24 hours) photosynthetic yield toxicity test to evaluate if this was a suitable approach to assess the toxicity of silver nanoparticles on algae. Such tests have been applied to silver nanoparticles to successfully determine their toxicity to
algae (Navarro et al., 2008; 2015; Miao et al., 2009; Dewez and Oukarroum, 2012; Oukarroum et al., 2012, Matorin et al., 2013). The work presented in this chapter evaluated two exposure protocols (a continuous 30 minute exposure, and longer term 4 and 24 hour exposures) developed for the investigation of toxicity of NM300K and ionic silver to R. subcapitata. Comparisons were made between growth inhibition studies in previous chapters, and to two commonly used reference substances (3-(3,4-dichlorophenyl)-1,1-dimethylurea and potassium dichromate) to validate toxicology studies in algae.
Chapter Two - Characterisation of silver nanoparticles

2.1. Introduction

Silver nanoparticles (NM300K) were obtained from the JRC of the European Commission (JRC). The JRC Nanomaterials Repository was established for research purposes in support of the OECD -

NM300K is a nano-silver colloidal dispersion with a nominal silver content of 10% w/w%, with non-ionic stabilizing agents (4% w/w% each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan mono-laurat (Tween 20)) (Klein et al., 2011). Particle size distribution is narrow (derived from scanning electron microscopy, transmission electron microscopy and nanoparticle tracking analysis), with the majority of particles having a diameter of around 15 nm, and a much smaller proportion around 5 nm (Klein et al., 2011). Vials contain an argon atmosphere to improve sample stability. Handling procedures advise vigorous shaking (4 minutes) of vials before use and that re-use after opening should be avoided to prevent nanoparticle aggregation/agglomeration. Due to the high viscosity of this reference material, a gravimetric method of determining quantity is recommended, to prevent overestimation due to the possibility of some of the nanoparticle suspension remaining in the pipette (Klein et al., 2011).

Two batches of this nanoparticle (NM300 and NM300K) were supplied by the JRC, as demand in the research community quickly exhausted the first batch (NM300). This thesis uses the second batch (NM300K), but both batches of nanoparticle were obtained from the same supplier (i.e. JRC), and were produced using the same initial material under a standard operating procedure. Therefore any reference to the characteristics of either batch is considered to be synonymously applicable between both batches (Klein et al., 2011). In addition, the JRC provides nanoparticle free dispersant vials (NM300K DIS; 4% w/w% each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan mono-laurat (Tween 20)) to be used to investigate potential toxicity of the dispersant in the desired test system.

Metal nanomaterials have properties which are distinct from both their dissolved and bulk (larger, not in the nanoscale) counterparts. Their unique physiochemical properties are what make them attractive to a variety of industries wanting to incorporate them into consumer products, however these properties may cause undesirable effects outside of their intended area of use (e.g. occupational exposure or environmental disposal) (Nowack and Bucheli, 2003; Kuhlbusch et al., 2011). In addition to verifying characterisation data provided by the nanomaterial supplier, robust characterisation data are important to identify which
nanomaterial properties may confer toxicity, and have the potential to steer industry towards the development of safer products using a “safety by design” approach (Geraci et al., 2015). Indeed characterisation data are essential in the understanding of nanomaterial fate, and thus exposure, and so important to understand and interpret hazard data. Many authors have attempted to provide guidelines, and differentiate between “essential” and “desirable” characterisation parameters (Oberdorster et al., 2005; Domingos et al., 2009; Klaine et al., 2008; Powers et al., 2006, 2007; Warheit 2008; Pettitt and Lead, 2013). In reality, such guidelines may be shaped by a variety of factors, such as the test system of interest (e.g. human cf. environment; test designs, including test species) and ultimate purpose of data gathered (fundamental research cf. regulation).

Due to the variety and diversity of nanomaterials, and the uncertainty of the principal mechanism(s) of action in different test systems, attempting to harmonise characterisation between studies is a high research priority. Some key nanomaterial properties have been suggested, which form a foundation for characterisation work in the specific context of REACH regulations (Pettitt and Lead, 2013; Table 2.1).

Table 2.1: Suggested primary properties of nanomaterial characterisation (adapted from Pettitt and Lead, 2013).

<table>
<thead>
<tr>
<th>Property</th>
<th>Key parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (pre and post exposure)</td>
<td>Mass, particle size distribution, particle number, surface area.</td>
</tr>
<tr>
<td>Core properties (pre and post exposure)</td>
<td>Elemental composition, chemical interactions, crystallinity, morphology, shape</td>
</tr>
<tr>
<td>Surface properties (pre and post exposure)</td>
<td>Specific surface area, charge, oxidation state, co-ordination chemistry, surface capping/coating agents</td>
</tr>
<tr>
<td>Aggregation characteristics (pre and post exposure)</td>
<td>Aggregate size and morphology, kinetics and mechanisms of aggregation/disaggregation</td>
</tr>
<tr>
<td>Solubility characteristics (metal nanomaterials; post exposure)</td>
<td>Dissolution rate, solubility</td>
</tr>
</tbody>
</table>

Silver nanomaterials are generally less toxic (on the basis of total silver concentration) than their ionic equivalents, to a number of organisms (see Bondarenko et al., 2013 for review). Slow silver ion release from nanomaterials is a property that is highly desirable for many applications, namely for their antimicrobial effects (Scientific Committee on Emerging
and Newly Identified Health Risks, 2014). There is uncertainty in the literature regarding the extent to which silver nanomaterial toxicity can be explained solely on the basis of dissolved silver ions (Klaine et al., 2008; Navarro et al., 2008; Miao et al., 2009; Wang et al., 2012; Gao et al., 2012; Xiu et al. 2012; Oukarroum et al., 2013; Sorensen and Baun, 2015; Gunsolus et al., 2015). Therefore accurately quantifying the dissolved silver fraction of a nanosilver suspension, preferably over test duration, is highly important in order to address such uncertainty (Kittler et al., 2010; Chappell et al., 2011; Sorensen and Baun, 2015), although this would not be able to provide full information on the conditions experienced by the exposed organism/model.

Solid silver (Ag\textsubscript{(0)}) is insoluble in water, but can gradually form various surface oxides (i.e. a surface layer of the metal which has reacted with oxygen – e.g. AgO, Ag\textsubscript{2}O, AgO\textsubscript{2}) (Chappell et al., 2011). These silver oxide species have low but non-trivial dissolution rates, and their production may be promoted in the presence of Reactive Oxygen Species (ROS) (Moore and Codella, 1998; Liu and Hurt, 2010; He et al., 2012) or at high dissolved oxygen concentrations (Metcalf and Harriott, 1972; Liu and Hurt, 2010). Xiu et al. (2012) found that PEG coated silver nanoparticles had no discernible dissolution when synthesised under anaerobic conditions. In the presence of oxygen, Ag\textsubscript{(0)} dissolution is also enhanced at lower pH levels (Peretyazhko et al., 2014; Liu and Hurt, 2010), higher temperatures (Liu and Hurt, 2010), and possibly in the presence of some non-ionic surfactants (Chappell et al., 2011). Liu and Hurt (2010) demonstrated that citrate-stabilised silver nanoparticles (< 10 nm) may re-adsorb dissolved Ag\textsuperscript{+} from the nanoparticle surface. Free Ag\textsuperscript{+} may also become bound to a number of compounds such as thiol groups of proteins (Miao et al., 2009; Szivak et al., 2009), humic acids (Liu and Hurt, 2010), EDTA (Chappell et al., 2011), and biogenic polymers (Miao et al., 2009). The stoichiometry of these different compounds will have a significant influence on silver speciation, solubility and bioavailability in both the natural environment and laboratory test systems.

Using NM300K as a representative silver nanoparticle is advantageous due to the fact that a considerable amount of standard and inter-laboratory characterisation has already been performed (Klein et al., 2011; Wang et al., 2012; Kermanizadeh et al., 2013; Voelker et al., 2013; Sorensen and Baun, 2015). Despite this, existing characterisation data may lose its relevance once NM300K is used in various test systems, where it may undergo different types of transformation. For example, Klein et al. (2011) used ICP-MS to characterise NM300K dissolution in three different media (18 mΩ water, 0.5 % HNO\textsubscript{3}, and “physiological phosphate buffer”). Dissolution was quantified as < 0.01 % of total silver, when NM300K was embedded in an acrylic matrix, presumably to facilitate later removal of the nanoparticles. Whilst this choice of methodology may improve sample handling, it has limited relevance to NM300K’s
use in hazard testing and/or its intended industrial applications. A number of studies have characterised NM300K properties in other tests media. Volker et al. (2013) found that in OECD M4 medium (Daphnia spp.) NM300K showed a dynamic light scattering (DLS) derived particle size of 57.6 ±1.2 nm and surface charge of -17 ±0.57 mV, and dissolution of < 2 % after 48 hours (using ultracentrifugation/ICP-MS). Kermanizadeh et al. (2013) found that in cell culture medium NM300K showed three DLS derived particle size classes of 12, 28, and 114 nm, and a dissolution of < 1 % in 18m water and C3A medium after 24 hours (using ultracentrifugation/AAS). Wang et al. (2012) found in unspecified media, NM300K exhibited a DLS derived zeta potential of -10 to -14 mV, a wide particle size distribution (100-200 nm), and a free Ag⁺ concentration of 1-10 % of total silver, over 96 hours (using ion-selective electrodes). Sorensen and Baun (2015) found that in OECD 201 medium (as used in this work) NM300K had a DLS derived size of 106 ±53 nm, a zeta potential of -21 ±4.3, a PDI of 0.15 ±0.045 (indicating monodispersity) at zero hours. Based on the nominal size of NM300K, Sorensen and Baun (2015) also calculated particle surface area to be 38 m²/g. Differences in these characterisation data are likely driven by tests system (including test medium), timepoint, preparation method (e.g. sonication), NM300K concentration and characterisation method. All existing studies have focussed on toxicologically relevant concentrations in the mg/l range (Wang et al., 2012; Kermanizadeh et al., 2013; Voelker et al., 2013; Sorensen and Baun, 2015). Despite two studies finding that certain particle properties (e.g. dissolution) are concentration dependent (Wang et al., 2012; Kermanizadeh et al., 2013), little data exist on NM300K properties in the µg/l range. Mallèvre et al. (2014) found that NM300K dissolution was not significantly different immediately after mixing in suspension and one hour later, in both Luria Bertani (bacterial) laboratory rich medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7) or in artificial wastewater (AW) consisting of Agrobacterium (AB) mineral medium (2 g/l (NH₄)₂SO₄, 6 g/l Na₂HPO₄, 3 g/l KH₂PO₄, 3 g/l NaCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 3 mM FeCl₃, pH 7) supplemented with 0.5% (w/v) of glucose as carbon source. Considering algae are some of the most sensitive test organisms to silver (Bondarenko et al., 2013), the sensitivity of R. subcapitata to NM300K is in the microgram per litre range (Chapter 3), and standard toxicity test duration is 72 hours (OECD, 2011), it is important to characterise nanomaterials (where possible) at the same concentration range, and at multiple timepoints.

### 2.1.1. Particle stabilisation

In order to prevent agglomeration/aggregation, nanomaterials can be stabilised to ensure a uniform particle size distribution. This can be achieved both chemically, through the addition of natural and synthetic ligands/polymers, and physically, through high energy mixing using ultrasonic frequencies (sonication). Commonly used chemical stabilisers in metal
nanomaterials include citrate (Jin et al., 2004; Guo et al., 2005; Cumberland and Lead, 2009; El Badawy et al., 2010; Thio et al., 2012; Gunsolus et al., 2015; Sorensen and Baun; 2015) and polyvinylpyrrolidone (PVP) (El Badawy et al., 2010; Thio et al., 2012; Wang et al., 2012; Gunsolus et al., 2015), due to their high stability, low toxicity and high solubility (Thio et al., 2012). Citrate stabilises silver nanomaterials electrostatically (based on surface charge), whereas PVP stabilises them sterically, through the adsorption of polymer to the surface forming a physical barrier (El Badawy et al., 2010).

Derjaguin, Landau, Verwey and Overbeek (DLVO) theory states that inter-particle electrostatic forces (as measured by DLS/zeta potential) greatly affect nanomaterial suspension stability, as nanoparticles with a uniformly high surface charge (over 30 mV, or below -30 mV) will repel one another within a suspension and confer system stability (Hanaor et al., 2012; Malvern Instruments, 2013). Whilst Klein et al. (2011) did not measure the surface charge of NM300K, subsequent studies have not found sufficient evidence to suggest that suspensions of this nanoparticle fit the true definition of DLVO electrostatic stability (i.e. ± 30 mV) (Wang et al., 2012; Volker et al., 2013; Johnston et al., 2015; Sorensen and Baun, 2015; Donnellan et al., 2016). However cross validation measurements have indicated that NM300K suspensions may exhibit high stability through other mechanisms. Wang et al. (2012) found that despite leaving suspensions of NM300K to settle for up to 96 hours, over 90% of total particulate silver (i.e. nanoparticles) remained in suspension. Wang et al. (2012) also tested two other silver nanoparticles (bare and PVP coated) of similar size, with higher zeta potential; these particles exhibited rapid sedimentation over the same time period, with less than 20% of total particulate silver in suspension. Soresen and Baun (2015) also recorded polydispersity index (PDI) and found that NM300K showed a narrow particle size distribution, indicating uniform dispersity with little aggregation (Malvern Instruments, 2013).

The addition of a non-ionic polymer (e.g. polyoxyethylene (20) sorbitan mono-laurat and polyoxyethylene glycerol trioleate, in the case of NM300K DIS) can improve the stability of nanomaterial suspensions, without affecting surface charge through a discrete process known as steric stabilisation (Napper, 1982). Non-ionic polymers have been demonstrated to improve stability of carbon nanotubes (Chappell et al. 2009) and silver nanoparticles (Chappell et al., 2011; El Badawy et al., 2010; Thio et al., 2012) in a variety of artificial media. Compared to electrostatic stabilisation, steric stabilisation has a number of advantages (Napper, 1982; Tauer, 2004; El Badawy et al., 2010), including:

- Insensitivity to media ionic strength/pH.
• Reversible flocculation; weakly bonded flocculation (if present) can be easily redispersed (e.g. by sonication).

• Concentration-independent stability; dispersions maintain their fluidity at high particle concentrations.

• Temperature-independent stability.

As a result, a sterically stable nanomaterial suspension can retain a high monodispersity within zeta potential ranges that would normally indicate electrostatically unstable dispersions (i.e. zeta potential of <30 mV, or > -30 mV).

There is some ambiguity within the literature regarding the description of stabilising agents (i.e. associated compounds added to nanomaterial suspensions to prevent aggregation/agglomeration). Within the limited studies of NM300K, three studies refer to the inclusion of non-ionic polymers as a “dispersion” (Klein et al., 2011; Wang et al., 2012; Sorensen and Baun, 2015), one as a “capping agent” (Kermanizadeh et al., 2013), one as containing “emulsifiers” (Johnston et al., 2015), and one incorrectly as “PVP coated” (Volker et al., 2013). Mallèvre et al. (2014) specifically refers to NM300K as “uncoated/in dispersion”. For the purposes of clarity in this thesis, dispersion will be used exclusively to refer to aqueous suspensions of nanomaterial and stabilising agents, without making assumptions regarding interactions between the two (i.e. stabilising agent is not assumed to be permanently bound to the associated nanoparticle). Terms such as coating or capping will only be used in cases when interactions between nanomaterial and stabilising agent have been documented (i.e. stabilising agent is demonstrably bound to the associated nanomaterial), in either liquid or solid mixtures. In the case of NM300K, terminology used by the JRC (describing batches as dispersions) will be adopted throughout this thesis (Klein et al., 2011).

Dispersion protocols involving mechanical processes (e.g. sonication/stirring) are often used to ensure nanomaterial suspensions retain a uniform particle size distribution and low agglomeration, immediately before use in toxicology tests (Ji et al., 2010; Wang et al., 2010; Taurozzi et al., 2011). For NM300K, documented sonication times ranged from ca. 15-20 minutes (Kermanizadeh et al., 2013; Mallèvre et al., 2014; Johnston et al., 2015; Sorensen and Baun, 2015; Donnellan et al., 2016) to 5 hours (Wang et al., 2012). Surprisingly, some studies have found that silver nanoparticle aggregation/agglomeration has little effect on reducing available surface area and resulting oxygen-mediated dissolution (Liu and Hurt, 2010; Zhang et al., 2011; Gunsolus et al., 2015). Therefore if ionic silver is the primary mechanism of toxicity,
differences in silver nanomaterial agglomeration/aggregation state may, in some specific cases, have little effect on toxicity.

2.1.2. Effects of media conditions: pH and humic acid

Medium composition, pH and humic acid were selected as relevant test conditions to modify exposure conditions and study their effects on toxicity (Chapter 3). Two different media (OECD and Jaworsky’s medium) were investigated in this thesis, to determine the effect of media choice on nanoparticle properties (Chapter 2) and resultant toxicity (Chapter 3). The chemical composition of each medium can be found in Appendix 1. OECD medium is one of the two standard media suggested for use in the OECD 201 Algal growth inhibition test (OECD, 2011). Jaworsky’s medium (JM) is a culture medium suggested by the Culture Collection of Algae and Protozoa for maintenance of R. subcapitata stock cultures (CCAP, 2014), and used in culture line maintenance at Heriot-Watt University. Comparing between the two media tested in this study, OECD medium has a lower ionic strength (1.67 mmol/l, compared to 4.08 mmol/l in JM) due to the lower concentrations of major (N, P, K and Na) and minor (B, Mn, Mo, Fe) elements. The influence of pH was selected for investigation in both media for two reasons; firstly OECD 201 test guidelines permit a 1.5 unit change over the 72 hour test duration (OECD, 2011) and it was therefore of interest to identify how such changes in pH may affect toxicity estimates, and secondly it adds a degree of environmental relevancy by establishing how a pH range similar to that expected in natural waters may modify toxicity. Some studies have shown that increases in ionic strength and changes in pH can cause electrostatically stable silver nanoparticles to aggregate and settle out of suspension (Cumberland and Lead, 2009; El Badawy et al., 2010; Delay et al., 2011). However sterically stable (PVP) silver nanoparticles were unaffected by media conditions such as ionic strength or pH in previous studies (e.g. El Badawy et al., 2010). Both media used in this study show marked differences in their chemical composition, which are likely to influence both algal growth and silver toxicity (ionic and nanoparticulate) (Chapter 3).

Organic matter was selected as a second parameter for investigation in both media. Suwannee River humic acid (SRHA), was used as a source of organic matter. By investigating humic substances, a greater degree of environmental realism can be achieved. In some cases, humic substances have been demonstrated to further stabilise electrostatically stable silver nanoparticles (Cumberland and Lead, 2009; Delay et al., 2010). Wang et al. (2015) found that humic acid reduced the toxicity of sterically stable PVP particles to R. subcapitata, Chydorus sphaericus and Danio rerio, but explained this mechanism through humic substance induced modification of surface charge (despite charge being a less significant mechanism of stabilising
PVP nanoparticles). In reality, few studies have investigated the effect of humic substances on sterically stable silver nanoparticle suspensions (e.g. NM300K).

2.1.3. Characterisation techniques

Understanding nanomaterial behaviour and toxicology, for both research and regulation purposes, requires accurate physico-chemical characterisation to be performed in parallel. Selection of characteristics to measure, and the methods by which to evaluate them, is still widely contested (Oberdorster et al., 2005; Domingos et al., 2009; Klaine et al., 2008; Powers et al., 2006, 2007; Warheit 2008; Pettitt and Lead, 2013). Characterising nanomaterials pose a variety of methodological issues, with multiple characterisation methods necessary to build a complete overview of particle properties such as dispersity, surface charge, particle size, dissolution and agglomeration/aggregation across a range of nanomaterial concentrations and in a variety of media over time (Domingos et al., 2009). However obtaining such comprehensive characterisation data is time consuming and complicated (Fabrega et al., 2011). Furthermore, if representative test materials are used, the need for extensive characterisation is reduced due to the implicit assumption of suitability in method validation due to previous extensive characterisation (Klein et al., 2011; International Organisation for Standardisation, 2013; Roebben et al., 2013). As a result, particle size, zeta potential, and dissolution were chosen as the main focus of this thesis. DLS offers rapid characterisation of both size and zeta potential in aquatic media, and is widely used for a variety of nanomaterials allowing robust comparisons to be made with existing studies. However DLS requires a minimum sample concentration in order to sufficiently scatter light (Malvern Instruments, 2013) which, in some cases may exceed toxicologically relevant concentrations (i.e. those used to generate concentration response curves) and is only applicable to spherical particles. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was chosen to study dissolution in this study, the latter at concentrations relevant to toxicology data. As NM300K (and its parent batch, NM300) is deemed stable in suspension, and has been well characterised across a variety of media/test systems (Klein et al., 2011; Wang et al., 2012; Kermanizadeh et al., 2013; Volker et al., 2013; Losasso et al., 2014; Mallèvre et al., 2014; Johnston et al., 2015; Sorensen and Baun, 2015; Donnellan et al., 2016), characterisation performed in this work aimed to characterise NM300K suspensions in media and conditions relevant to the hazard studies conducted and to compare results obtained to findings of previous authors.

*Inductively Coupled Plasma – Mass Spectrometry (ICP-MS)*

ICP-MS is used to measure the concentration of metal ions in a solution, but with a much lower limit of detection than AAS. As such, it is much better suited for measuring
dissolved metal at low concentrations. ICP-MS has been used to study the solubility of metal nanomaterials, and has been demonstrated to be suitable for detecting silver, titanium and cerium in the ng/l range (Reed et al., 2012). Ultracentrifugal filtration is used to measure the dissolved metal fraction in nanomaterial suspensions. However some authors have questioned whether such methods underestimate dissolved silver due to losses of ions to filtration units (Kennedy et al., 2010; Dong et al., 2016). A low limit of detection provided by ICP-MS is particularly important when attempting to characterise dissolution at test condition relevant concentrations, where the test organism may be highly sensitive (e.g. algae – Bondarenko et al., 2013). According to published literature, NM300K appears to have low (< 10 %) dissolution in experimental media (Klein et al., 2011; Wang et al., 2012; Kermanizadeh et al., 2013; Volker et al., 2013; Mallèvre et al., 2014; Sorensen and Baun, 2015), which places further emphasis on the requirement for a low limit of detection.

**Dynamic Light Scattering**

DLS is a method used to measure the size and number of nanoparticles in a suspension. A laser is used to measure the fluctuations in light-scattering of the particles over time, as a result of their Brownian motion. By measuring changes in Brownian-induced differences in light scatter of the sample, particle size-distribution of a solution can be determined (Malvern Instruments, 2013).

As each individual particle in a suspension moves independently, the intensity of the scattered light reaching the detector will fluctuate over very short time periods (micro-nanoseconds). Over time the signal pattern will become increasingly different from the first measurement taken upon introduction of the sample to the machine (i.e. t₀), as the particles in suspension rearrange their position relative to each other and the laser beam. By correlating the differences in signal over time, the size of the particles can be determined. According to Stokes-Einstein, the rate at which the correlation between two signals is reduced to zero is directly proportional to particle size. Samples containing smaller particles will become quickly dissimilar due to the rapid Brownian motion, whereas samples containing larger particles will take longer to become dissimilar, as they will move more slowly (Malvern Instruments, 2013).

One limitation of this method is that the Stokes-Einstein equation assumes that the particles are spherical and do not interact with each other. Therefore this method may only be reliably used for roughly spherical, non-aggregating particles. Size data on nanomaterial suspensions that deviate from these assumptions (e.g. non spherical nanomaterials such as nanorods, or polydisperse suspensions as a result of complex media interactions) may best be derived from other methods (Reed et al., 2010). Furthermore, high particle concentrations (> 1
mg/l) are needed to sufficiently scatter light and generate adequate results (Domingos et al., 2009).

Many studies use DLS and refer to “particle diameter”, though this is slightly misleading. 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. As such, hydrodynamic diameter (measured by DLS) is sensitive to ionic strength and other medium specific factors (Jiang et al., 2009). Of the studies which evaluated NM300K particle size using DLS, hydrodynamic diameter has been found to be larger than the bare particle size of 15 nm described by Klein et al. (2011). Hydrodynamic diameter of 12-140 nm have been reported (Kermanizadeh et al., 2013; Volker et al., 2013; Losasso et al., 2014; Mallèvre et al., 2014; Sorensen and Baun, 2015; Donnellan et al., 2016), which may vary due to medium composition or dispersion protocol. DLS has an added disadvantage that it is non-specific with regards to particle detection, that is it is unable to distinguish between different particles and so may be influenced by the presence of non-target particulate matter (e.g. humic substances, incidental dust introduced during sample preparation, etc. – Reed et al., 2012; Malvern Instruments, 2013)

**Zeta potential**

Zeta potential is the charge potential of a particle at the boundary within the diffuse layer, where ions closer to the particle remain bound as the particle moves, and ions further from the particle remain unbound. By applying an electric field across a particle suspension, the speed of the responding charged particle movement can be measured to derive the electrophoretic mobility. Particle electrophoretic mobility is a function of applied electric field strength, medium viscosity, medium dielectric constant, and zeta potential (Malvern instruments, 2016). Therefore if these media properties are well defined, zeta potential can be easily derived. Incidentally, as zeta potential depends highly upon medium properties (temperature, pH, ionic strength); comparing zeta potential between nanoparticles is not useful unless the same suspension conditions are maintained (Malvern instruments, 2016).

For the purposes of discussion in this thesis, any reference to changes in zeta potential will refer specifically to surface charge density. Therefore any increase in zeta potential should be read as a departure from zero charge, either positive or negative. In situations where context is unclear, more information will be provided.
2.2. Aims and Objectives

The aim of the work presented in this chapter was to investigate the hydrodynamic diameter, zeta potential and solubility of NM300K nanoparticles in water and algal medium. To achieve this, the following objectives were identified:

- To investigate the solubility of NM300K in water and algal medium (OECD and JM), under OECD 201 test conditions (algae free; Chapter 3). ICP-MS solubility studies were carried out in both media, at concentrations toxicologically relevant to the inhibitory concentration of NM300K to *Raphidocelis subcapitata* [100 µg/l Ag], at 0 and 72 hours. For reference to other published literature (and comparison between methods), ICP-MS [100 µg/l Ag] was carried out in 18 mΩ water.

- To investigate the effect of pH and humic acid on NM300K dissolution (in OECD and JM), under modified (with humic acid and pH) OECD 201 test conditions (algae-free; Chapter 3). ICP-MS solubility studies were carried out at 100 µg/l Ag NM300K in both media at 0 and 72 hours. pH was modified to 6 and 8, using NaOH, HCl and 3.5 mM MOPS buffer. Humic acid was modified using Suwannee River humic acid, at 5 and 50 mg/l SRHA.

- To investigate the solubility of freshly opened and aged vials of NM300K. ICP-MS solubility studies were carried out in 18 mΩ water at 100 µg/l Ag, using a single vial of freshly opened NM300K and a single vial of NM300K which had been opened and stored for one year, in the dark at room temperature.

- To investigate stability of NM300K in water and algal medium (OECD medium and JM) via assessment of hydrodynamic diameter, zeta potential and polydispersity index using DLS. These studies were carried out at 5 and 10 mg/l Ag NM300K, at 0 hours.

- To investigate the effect of humic acid on NM300K stability (in 18 mΩ water, OECD medium and JM). DLS studies were carried out at 5 and 10 mg/l Ag NM300K, at 0 hours. Humic acid will be modified using Suwannee River humic acid, at 5 and 50 mg/l SRHA.
2.3. Hypotheses

Research hypotheses

- NM300K dissolution will be low (< 10%) in 18 mΩ water and algal media. The composition, pH and presence of humic acid will impact of silver nanoparticle dissolution. More specifically lower pH will enhance dissolution and the presence of humic acid will reduce the amount of dissolved silver. NM300K dissolution will also increase over time.

- A freshly opened vial of NM300K will exhibit lower dissolved silver than a vial which has been open for one year.

- NM300K stability (hydrodynamic diameter, zeta potential, and polydispersity index) will be unaffected by media composition (OECD medium or JM), due to steric stabilisation.

- Humic acid will increase particle hydrodynamic diameter, but the NM300K suspension will remain polydisperse and stable.

Null hypotheses

- NM300K dissolution will be unaffected by medium parameters such as elemental composition, pH or humic acid.

- There will be no difference in dissolved silver between a freshly opened and a one year old vials of NM300K.

- NM300K stability (hydrodynamic diameter, zeta potential, and polydispersity index) will be affected by media composition (OECD medium or JM).

2.4. Methods

2.4.1. Glassware and toxicant stocks

Glassware for toxicant preparation was pre-washed in 50 % nitric acid (HNO₃) for 15 minutes, then rinsed four times in distilled water and air dried. Glassware for media preparation (toxicant free) was washed with a polyurethane sponge and a 5 % (v/v) solution of Decon 90 (Decon Laboratories, LTD), and then rinsed four times in distilled water and air dried.
Vials of silver nanoparticles suspended in a dispersant (NM300K – 10.16 % w/w AgNP) were provided by the Joint Research Centre. All stock suspensions/solutions were prepared on the same day of testing.

Working master stock suspensions of silver nanoparticles were prepared on the day of testing to a final target concentration of 100 mg/l Ag (w/v). First 49.2 mg of NM300K dispersion was weighed, and added to 25 ml 18 mΩ water in a 50 ml borosilicate volumetric flask. This 25ml suspension was sonicated at 38 kHz ± 10 % in a bath sonicator (Pulsatron 325, Kerry) for two 8 minute bursts, with ~10 seconds manual shaking in between (volumetric flasks were partially filled to 25 ml before sonication to allow adequate dispersal of particles). After sonication, flasks were filled to the 50 ml mark with 18 mΩ water, to reach the final concentration of 100 mg/l Ag (w/v), and shaken vigorously. Stock suspensions of lower NM300K concentration (typically 10 mg/l Ag) were created from master stocks by serially diluting the master stocks to the required concentration in 50 ml volumetric flasks and manually shaking for ~1 minute.

2.4.2. Assessment of nanoparticle dissolution using ICP-MS

2.4.2.1. The effect of media

The concentration of NM300K particles in 2 ml vials, supplied by the JRC was 10.16 % Ag (w/w). NM300K nanoparticles were diluted to a concentration of 10 mg/l Ag in 18 mΩ water (to create a working stock suspension as described in Section 2.4.1.). NM300K stock suspensions were prepared in acid-washed 50 ml volumetric flasks (Fisher Scientific). Final NM300K test concentrations were 100 µg/l Ag in all test conditions.

For unmodified medium (OECD medium or JM) this comprised of 0.9 ml of the NM300K stock suspension [10 mg/l Ag], 21.6 ml 18 mΩ water, 22.5 ml double strength medium, and 45 ml standard strength medium (for details of the composition of each medium refer to Appendix 1).

For pH modified medium, two 0.5 M MOPS (3-(N-morpholino)propanesulfonic acid; C₇H₁₅NO₄S; used as buffer) stocks were prepared in 18 mΩ water, and adjusted to pH 6 and 8 respectively, using NaOH or HCl. MOPS stocks were syringe filtered (pore size - 0.2 µm) into sterile acid-washed glass bottles, and used as pH stocks for all subsequent pH experiments. A volume of 0.63 ml 0.5 M MOPS solution (at pH 6 or 8) was added to 15 ml 18 mΩ water, 22.5 ml double strength medium, and 45 ml standard strength medium. Solutions were adjusted to the either pH 6 or 8 using HCl and NaOH solutions (final MOPS concentration: 3.5 mM). Volumes of added NaOH and HCl were recorded for each test; pH was measured using a probe
(Orion ROSS Ultra, Thermoscientific) connected to an electrochemistry meter (Orion Star Plus, Thermo Scientific) which was calibrated on the day of use. A volume of 0.9 ml of the NM300K stock suspension [10 mg/l Ag] was added to the buffered media solutions, and made up to a final volume of 90 ml.

For humic acid modified medium, Suwannee River humic acid stock solutions were prepared at a concentration of 1 g/l (w/v) in 18 mΩ water in a sterile acid washed 50 ml volumetric flask. To prepare a 5 mg/l SRHA suspension of NM300K particles, 0.9 ml of the SRHA stock solution [1 g/l], was added to 0.9 ml of the NM300K stock suspension [10 mg/l Ag], 20.7 ml 18 mΩ water, 22.5 ml double strength medium, and 45 ml standard strength medium. To prepare the 50 mg/l SRHA suspension of NM300K particles, 9 ml of SRHA stock [1 g/l], 0.9 ml of the NM300K stock suspension [10 mg/l Ag], 12.6 ml 18 mΩ water, 25 ml double strength medium, and 45 ml standard strength medium.

From each of these 90 ml NM300K suspensions, 15 ml volumes were aliquoted into six acid-washed 20 ml glass vials. At t₀, 10 ml of each sample was removed from three of the vials, centrifuged and acidified using the protocol described below. The remaining samples were sealed with a foam bung and aged for 72 hours in the test system, by placing in an orbital shaker (Multitron Standard, Infors-HT), at 225 RPM, under continuous fluorescent light ~120 µmol/m²/s, at 23˚C ± 2. After 72 hours in the test system, the remaining samples were also removed and prepared for analysis. The remaining 5 ml in each vial was discarded.

In order to remove nanoparticles from suspension and quantify dissolved silver, 10 ml of each of the nanoparticle suspensions were aliquoted into 10 ml open top polycarbonate centrifuge tubes (Beckman Coulter, USA) in triplicate. Using a method adapted from previous studies (Li et al., 2011; Zook et al., 2011; Mallèvre et al., 2014) samples were centrifuged at 60,200-74,200 g (depending on placement of tube in centrifuge rotor) for 60 minutes at 20°C (Avanti J-25 ultracentrifuge; JA 25.15 rotor; Beckman Coulter, USA). HNO₃ (0.9 M, 5ml) was added to 5 ml of the supernatant for each sample, for analysis of dissolved silver. To test recovery (i.e. dissolved silver + particulate silver) in a limited set of samples, the remaining 5 ml of supernatant and the nanoparticulate pellet were acid digested using 5 ml of a 15.6 M solution of HNO₃, to dissolve particulate silver and obtain an ionic solution. These dissolved particulate silver solutions were diluted by taking 0.4 ml and adding to 9.6 ml 18 mΩ water to create a constant HNO₃ concentration in all samples (i.e. dissolved and particulate; 0.45 M).

Dissolution data were obtained by measuring the ^107Ag isotope using ICP-MS, as described in Verleysen et al. (2015). Briefly, the concentration of silver in the supernatant and pellet was measured at 0 and 72 hours post preparation and incubation in the test system,
using ICP-MS at the University of Edinburgh under the supervision of Dr Lorna Eades. An Agilent 7500ce (with octopole reaction system) ICP-MS was used, employing an radio frequency forward power of 1540 W and reflected power of 1 W, with argon gas flows of 0.81 l/min and 0.19 l/min for carrier and makeup flows, respectively. Sample solutions were taken up into the Micro mist nebuliser by free aspiration at a rate of approximately 1 ml/min. Skimmer and sample cones were made of nickel.

The instrument was operated in spectrum acquisition mode and three replicate runs per sample were employed. The mass was analysed in fully quant mode (three points per unit mass) and analysed in standard ‘no gas’ mode. To minimise interference from adherent effects of silver between samples an additional base rinse step was used (a mixture of NH₃, EDTA, H₂O₂ and Triton x-100).

A calibration curve was obtained at the beginning of every sample run, using AgNO₃ as a standard (0.01, 0.025, 0.1, 0.25, 1, 2.5, 10, 25, 100 µg/l Ag). An internal standard of rhodium (¹⁰³Rh 20 µg/l) was included in all samples to monitor changes in machine sensitivity over the sample run, and a check concentration of 2.5 µg/l was included between every 12 samples. An external reference standard SRM1640a (National Institute of Standards and Technology, USA) was diluted 100 and 10 fold to check for accuracy of the standard curve.

### 2.4.1.2. The effect of sample vial age

In addition to investigating NM300K properties when dispersed in different media, a pilot study was carried out to investigate potential dissolution in the original sample vials over time (triplicate samples of two single vials). Stock silver nanoparticle suspensions were prepared from two NM300K vials as described above; one which had been opened on the day of experiment, and one which had been opened and then stored in the dark at 20°C for 12 months (termed aged nanoparticles). NM300K stock suspensions [10 mg/l Ag] of fresh and aged nanoparticles were prepared as described above, in 18 mΩ water. Test concentrations were prepared at 100 µg/l Ag NM300K in 18 mΩ water, by adding 0.45 ml NM300K [10 mg/l Ag] to 44.55 ml 18 mΩ water, and aliquoting into 15 ml volumes into three acid-washed 20 ml glass vials. Samples were then centrifuged and measured using ICP-MS as described above.

### 2.4.3. Measurement of nanoparticle hydrodynamic diameter and zeta potential

NM300K stock suspension [100 mg/l Ag] was diluted in double-strength algal growth medium (OECD medium and JM) to concentrations of 5 and 10 mg/l Ag. Each concentration was made in triplicate 15 ml volumes for each medium, following the procedure described in ICP-MS sample preparation (Section 2.4.2.). Nanoparticle suspensions (5 and 10 mg/l Ag) were
prepared in 45 ml volumes. For unmodified medium (OECD medium or JM) this comprised of 2.25 ml or 4.5 ml of the NM300K stock suspension [100 mg/l Ag], 9.25 ml or 6.75 ml 18 mΩ water, 11.25 ml double strength medium, and 22.5 ml standard strength medium. Samples were prepared, then aliquoted into three in acid washed 20 ml glass vials. For humic acid modified medium, Suwannee River humic acid stock solutions were prepared at a concentration of 1 g/l (w/v) in 18 mΩ water in a sterile acid washed 50 ml volumetric flask using the same sonication protocol described for NM300K (Section 2.4.1.). This was then added to the unmodified media to prepare 45 ml suspensions for each NM300K concentration (5 and 10 mg/l Ag); at 5 and 50 mg/l SRHA. For 5 mg/l Ag NM300K with 5 mg/l SRHA, 2.25 ml of NM300K suspension [100 mg/l Ag] was combined with 0.225 ml SRHA stock [1 g/l], 8.775 ml 18 mΩ water, 11.25 ml double strength medium, and 22.5 ml standard strength medium. For 5 mg/l Ag NM300K with 50 mg/l SRHA, 2.25 ml of NM300K suspension [100 mg/l Ag] was combined with 2.25 ml SRHA stock [1 g/l], 6.25 ml 18 mΩ water, 11.25 ml double strength medium, and 22.5 ml standard strength medium. For 10 mg/l Ag NM300K with 5 mg/l SRHA, 4.5 ml of NM300K suspension [100 mg/l Ag] was combined with 0.225 ml SRHA stock [1 g/l], 6.525 ml 18 mΩ water, 11.25 ml double strength medium, and 22.5 ml standard strength medium. Finally, for 10 mg/l Ag NM300K with 50 mg/l SRHA, 4.5 ml of NM300K suspension [100 mg/l Ag] was combined with 2.25 ml SRHA stock [1 g/l], 4.5 ml 18 mΩ water, 11.25 ml double strength medium, and 22.5 ml standard strength medium. From each of these 45 ml master suspensions, 15 ml volumes were aliquoted into three acid-washed 20 ml glass vials. All samples were measured immediately after preparation.

Dynamic Light Scattering (DLS, Malvern Zeta Sizer Nano Series) was used to measure hydrodynamic diameter, zeta potential, and polydispersity index (PDI). Samples were analysed in a “Size & zeta potential” Folded Capillary cell (DTS1060, Malvern Instruments). Each sample was prepared immediately before measurement, and acclimated to 22°C before measurements were taken.

2.4.4. Statistical analysis

Data were analysed in Minitab 17 statistical software, with a significance level of 5 % (p < 0.05). When data were found to be normally distributed, or could be transformed to fit a normal distribution, parametric statistics were used. For non-normally distributed data, non-parametric equivalent tests were used. The statistical tests used were one way ANOVA (for normally distributed/transformed data), Tukey’s post hoc test (to identify significant differences between > 2 groups), Kruskal-Wallis test (for non-normal data), Mann-Whitney U post hoc test (used to make multiple pairwise comparisons in non-normal data for > 2 groups;
or to make single pairwise comparisons between two non-normally distributed groups). For comparisons between two normally distributed data sets a student’s T test was used. Tests deployed were indicated in the results section with the appropriate test statistic; F (One way ANOVA), H (Kruskal-Wallis), W (Mann-Whitney U), and T (students T-test).

2.5. Results

2.5.1. Assessment of nanoparticle dissolution using ICP-MS

2.5.1.1. The effect of media

The impact of medium composition, humic acid and pH on NM300K [100 µg/l Ag] dissolution was investigated (Table 2.2; Figures 2.1-2.3). As 100 µg Ag NM300K/l was used, percentage silver dissolution (%) and absolute silver dissolution (µg/l Ag) can be used interchangeably; for comparison with published literature all dissolution data was expressed as percentage.

Whilst NM300K dissolution appeared to increase over time for all OECD medium conditions (Table 2.2), significant time dependent dissolution was only observed in unmodified medium (T = -5.47, p = 0.032), medium containing 50 mg/l SRHA (T = -19.06, p = 0.003), and medium buffered to pH 6 (T = -11.9, p = 0.007) (Table 2.2). Similarly for JM significant time dependent increases in NM300K dissolution were only observed in medium containing 5 mg/l SRHA (T = -9.52, p = 0.002), 50 mg/l SRHA (T = -29.5, p = 0.002), and medium buffered to pH 6 (T = -14.59, p = 0.005) (Table 2.2). This therefore suggests that the presence of SRHA and a pH of 6 enhances NM300K dissolution. In JM buffered to pH 8, a significant decrease in NM300K dissolution was observed between 0 and 72 hours (T = 8.02, p = 0.004).
Table 2.2: ICP-MS percentage dissolved silver concentration of NM300K [100 µg/l Ag] in unmodified OECD medium and JM, medium buffered to pH 6 and 8, and medium with 5 and 50 mg/l Suwannee River humic acid (SRHA). Dissolution was measured after 0 and 72 hours of cell-free incubation using ICP-MS. Data expressed as mean dissolved silver and standard error of the mean (n = 1 one experiment with three replicates; except for OECD medium, 0 h, 50 mg/l SRHA where one replicate was lost). Superscript letters denote significant differences in NM300K dissolution between each timepoint within each media condition (Two sample t-test; p < 0.05); NM300K dissolution values not sharing the same superscript letter were significantly different. A source of t₀ dissolution variation was identified as an artefact of sample preparation (due to batch preparation of samples); therefore such treatments may not represent true t₀ values. This may also explain the apparent relative reduction in time dependent dissolution in JM pH 8, as an artefact.

<table>
<thead>
<tr>
<th>OECD medium</th>
<th>Unmodified</th>
<th>+ 5 mg/l SRHA</th>
<th>+ 50 mg/l SRHA</th>
<th>pH 6</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>2.44 (±0.04)ᴬ</td>
<td>8.42 (±0.68)ᴮ</td>
<td>1.72 (±0.04)ᴮ</td>
<td>2.39 (±0.08)ᴮ</td>
<td>3.83 (±0.15)ᴬ</td>
</tr>
<tr>
<td>72 h</td>
<td>4.10 (±0.30)ᴬ</td>
<td>12.29 (±1.62)ᴬ</td>
<td>44.72 (±2.22)ᴬ</td>
<td>6.08 (±0.30)ᴬ</td>
<td>4.35 (±0.20)ᴬ</td>
</tr>
<tr>
<td>JM</td>
<td>4.12 (±0.11)ᴬ</td>
<td>6.90 (±0.56)ᴮ</td>
<td>6.18 (±0.38)ᴮ</td>
<td>3.78 (±0.03)ᴮ</td>
<td>3.42 (±0.12)ᴬ</td>
</tr>
<tr>
<td>72 h</td>
<td>5.42 (±0.28)ᴬ</td>
<td>14.65 (±0.59)ᴬ</td>
<td>25.61 (±0.54)ᴬ</td>
<td>6.33 (±0.17)ᴬ</td>
<td>2.18 (±0.10)ᴮ</td>
</tr>
</tbody>
</table>
In unmodified media, NM300K dissolution was significantly higher in JM compared to OECD medium at both 0 (T = 13.96, p = 0.005) and 72 (T = 3.2, p = 0.049) hours (Figure 2.1).

**Figure 2.1**: ICP-MS percentage dissolved silver concentration of NM300K [100 µg/l Ag] in unmodified OECD medium (black bars) and JM (white bars). Dissolution was recorded after 0 and 72 hours of cell-free incubation. Data expressed as mean dissolved silver and standard error of the mean, (n = 1, one experiment with three replicates). Letters denote significant differences in NM300K dissolution between media within each timepoint (Two sample t-test; p < 0.05); NM300K dissolution values not sharing the same letter were significantly different.

The addition of SRHA tended to increase NM300K dissolution in both media relative to the corresponding unmodified media (Table 2.2, Figure 2.2). In OECD medium at 0 hours, a concentration of 5 mg/l SRHA caused a significant increase in dissolved silver relative to unmodified medium or medium containing 50 mg/l SRHA (F = 67.44, p < 0.001). By 72 hours, dissolved silver was significantly higher compared to earlier timepoints, and with increasing SRHA concentration (F = 181.68, p < 0.001) Comparatively, in JM at 0 hours, both 5 and 50 mg/l SRHA caused a significant increase in dissolved silver compared to unmodified medium (F = 13.26, p = 0.006). Similarly to OECD medium, in JM at 72 hours dissolved silver was significantly higher with increasing SRHA concentration (F = 431.3, p < 0.001). Across both humic acid concentrations and timepoints studied, significant differences in dissolved silver were observed only between media containing 50 mg/l SRHA (Figure 2.2); at 0 hours NM300K dissolution was significantly higher in JM (T = 11.55, p = 0.007), whereas by 72 hours OECD
medium exhibited significantly higher dissolved silver ($T = -8.37$, $p = 0.014$). Considered together, these data suggest that the presence of SHRA in both media types enhances NM300K dissolution.

Figure 2.2: ICP-MS percentage dissolved silver concentration of NM300K [100 µg/l Ag] in OECD medium (black bars) and JM (white bars), with the addition of 5 and 50 mg/l Suwannee River humic acid (SRHA). Dissolution was measured after 0 and 72 hours of cell-free incubation. Data expressed as mean dissolved silver and standard error of the mean ($n = 1$, one experiment with three replicates; except for OECD medium, 0 h, 50 mg/l SRHA where one replicate was lost). Letters denote significant differences in NM300K dissolution between media within each timepoint (Two sample t-test; $p < 0.05$); NM300K dissolution values not sharing the same letter were significantly different.

Compared to SRHA, the effect of pH modification on NM300K dissolution was lower in both media (Table 2.2, Figure 2.3). In OECD medium at 0 hours, only medium buffered to pH 8 showed a significant increase in dissolved silver relative to either unbuffered medium or medium buffered to pH 6 ($F = 64.45$, $p < 0.001$). At 72 hours, dissolved silver was only significantly higher in OECD medium buffered to pH 6 relative to either unbuffered medium or medium buffered to pH 8 ($F = 16.02$, $p = 0.004$). In JM at 0 hours, dissolved silver was significantly higher in unbuffered medium relative to medium buffered to pH 8 ($F = 13.68$, $p = 0.006$). At the same timepoint, pH 6 medium was not significantly different from either unbuffered or pH 8 test conditions. In JM at 72 hours, significant differences in all three pH conditions were observed ($F = 118.45$, $p < 0.001$). Across both pH conditions and timepoints studied, significant differences in dissolved silver were observed between media at 0 hours for
pH 6 (T = 15.9, p = 0.004) and 72 hours for pH 8 (T = -9.77, p = 0.002) (Figure 2.3). At 0 hours in media buffered to pH 6 NM300K dissolution was significantly higher in JM, but at 72 hours in media buffered to pH 8 OECD medium exhibited significantly higher dissolved silver. In summary there were no clear patterns regarding the influence of pH on silver dissolution across both media, OECD and JM.

![Graph](image)

**Figure 2.3:** ICP-MS percentage dissolved silver concentration of NM300K [100 µg/l Ag] in OECD medium (black bars) and JM (white bars), buffered to pH 6 and pH 8 using 3-(N-morpholino)propanesulfonic acid. Dissolution was recorded after 0 and 72 hours of cell-free incubation in test chamber. Data expressed as mean dissolved silver and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences in NM300K dissolution between media within each timepoint (Two sample t-test; p < 0.05); NM300K dissolution values not sharing the same letter were significantly different.

Recovery was investigated by comparing the silver concentration of both the supernatant and ultracentrifuged nanoparticle pellet with the nominal silver concentration expected in the test system [100 µg/l Ag], for four representative samples. Four single samples were chosen to test recovery; freshly opened NM300K, one year old NM300K, and NM300K in standard OECD medium before and after incubation in test system described above (Figure 2.4). As single measurements were taken, descriptions of results are tentative. Data of dissolved silver (ultracentrifuged supernatant) and particulate silver (acid digested pellet) fractions were added together to check recovery between the samples was consistent. Freshly
opened NM300K showed above 100% recovery, whereas year old NM300K and 0 hour NM300K (freshly opened NM300K) in OECD medium showed 98.84 % and 91.1 % recovery, respectively. For the NM300K sample (freshly opened) that had been prepared in OECD medium and incubated in the test system for 72 hours, recovery was only 33.49 %.

![Figure 2.4](image.png)

**Figure 2.4**: Recovery of four individual ICP-MS samples (experiment without replication). Total recovered silver shown as the addition of dissolved supernatant (black bars) and ultracentrifuged particulate silver (white bars). Apparent > 100 % recovery observed in fresh 0 h treatment identified as a likely artefact of sample preparation.

2.5.1.2. The effect of sample vial age

The impact of age of NM300K (as supplied by the JRC) on dissolution was investigated (Figure 2.5). Two samples were prepared in 18mΩ water; one using a freshly opened vial of NM300K, and one using a one year old (since opening) vial. Samples were centrifuged immediately after preparation. No significant difference in dissolved silver was observed between freshly opened vials and vials which had been open for one year, where NM300K dissolution was 3.79 (±0.39) % in the freshly opened vial and 3.7 (±0.43) % in the one year old vial.
Figure 2.5: ICP-MS percentage dissolved silver concentration in a single freshly opened vial of NM300K (black bars) and a single vial of NM300K opened one year previously, and used in routine toxicology experiments (white bars). All samples were diluted to 100 µg/l in 18mΩ water and sonicated. Data expressed as mean dissolved silver and standard error of the mean (n = 1, one experiment with three replicates).

2.5.2. Measurement of nanoparticle hydrodynamic diameter and zeta potential

DLS was used to measure NM300K particle hydrodynamic diameter, zeta potential and polydispersity index (PDI) when nanoparticles were dispersed in different media (OECD medium and JM; with and without humic acid modification), at different NM300K concentrations (5 and 10 mg/l Ag). While a range of NM300K concentrations (1.25, 2.5, 5 and 10 mg/l Ag) were originally studied in both media, high variation in the data obtained for nanoparticle concentrations below 5 mg/l Ag were attributed to testing concentrations below the limit of instrument detection. As a result, only 5 and 10 mg/l Ag were used for DLS analysis (Table 2.3).
Table 2.3: DLS NM300K [5 and 10 mg/l Ag] characteristics in unmodified OECD medium and JM, and medium with 5 and 50 mg/l Suwannee River humic acid (SRHA) added. Hydrodynamic diameter, zeta potential and polydispersity index were measured immediately after NM300K sample preparation. DLS data are expressed as mean and standard error of the mean (n = 1, one experiment with six combined replicates; three for 5 mg/l and three for 10 mg/l Ag NM300K). Superscript letters denote significant differences in NM300K characteristics between Suwannee River humic acid concentrations (One way ANOVA/Kruskal-Wallis; p < 0.05); NM300K characteristics not sharing the same superscript letter were significantly different.

<table>
<thead>
<tr>
<th>Medium conditions</th>
<th>OECD medium</th>
<th>+ 5 mg/l SRHA</th>
<th>+ 50 mg/l SRHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic diameter (nm)</td>
<td>28.48 (±1.97)$^B$</td>
<td>42.48 (±5.44)$^A$</td>
<td>41.72 (±2.36)$^{AB}$</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-16.05 (±1.81)$^A$</td>
<td>-15.97 (±1.94)$^A$</td>
<td>-17.85 (±0.86)$^A$</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.54 (±0.05)$^A$</td>
<td>0.48 (±0.002)$^A$</td>
<td>0.47 (±0.008)$^A$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medium conditions</th>
<th>JM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic diameter (nm)</td>
<td>31.95 (±3.01)$^C$</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-4.46 (±0.43)$^A$</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.47 (±0.02)$^A$</td>
</tr>
</tbody>
</table>

NM300K particle hydrodynamic diameter was < 60 nm in all media tested (Table 2.3, Figure 2.6). In 18 mΩ water the hydrodynamic diameter of NM300K particles was 31.43 (±2.07) nm. The presence of SRHA (5 and 50 mg/l) caused a slight increase in hydrodynamic diameter in both media. SRHA significantly increased NM300K particle size in both OECD medium (F = 4.75, p = 0.025) and JM (H = 11.61, p = 0.003). When comparing between media across humic acid concentrations (Figure 2.6), significant differences in hydrodynamic diameter were only observed at 50 mg/l SRHA (T = 3.08, p = 0.018), where a higher NM300K particle size was observed in JM. These results therefore demonstrate that in the presence of SRHA the hydrodynamic diameter of NM300K particles increases.
Figure 2.6: DLS hydrodynamic diameter of NM300K [5 and 10 mg/l Ag] in OECD medium (black bars) and JM (white bars), in the presence of 5 and 50 mg/l Suwannee River humic acid (SRHA). Hydrodynamic diameter was measured immediately after sample preparation. Data expressed as mean hydrodynamic diameter and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences in NM300K hydrodynamic diameter between media within each SRHA level (Mann-Whitney U/Two sample t-test; p < 0.05); NM300K hydrodynamic diameter values not sharing the same letter were significantly different.

NM300K zeta potential was between ±30 mV in both media (Table 2.3, Figure 2.7), and 18 mΩ water (-2.9 ±0.34 mV). In OECD medium, NM300K zeta potential was not significantly influenced by SRHA. Conversely in JM, NM300K zeta potential was significantly more negative with increasing SRHA concentration (F = 36.42, p < 0.001). When comparing between media across humic acid concentrations (Figure 2.7), significant differences in zeta potential were observed in both unmodified media (T = 6.24, p = 0.002) and media containing 5 mg/l SRHA (T = 4, p = 0.01), where a more negative NM300K surface charge was observed in OECD medium. By 50 mg/l SRHA, no significant difference in NM300K zeta potential was observed. These results demonstrate that in OECD medium, the presence of SRHA has no effect on the zeta potential of NM300K particles. Conversely in JM, the zeta potential of NM300K particles became more negative in the presence of SRHA.
Figure 2.7: DLS zeta potential of NM300K [5 and 10 mg/l Ag] in OECD medium (black bars) and JM (white bars), with the addition of 5 and 50 mg/l Suwannee River humic acid. Zeta potential was measured immediately after sample preparation. Data expressed as mean zeta potential and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences in NM300K zeta potential between media within each SRHA level (Two sample t-test; p < 0.05); NM300K zeta potential values not sharing the same letter were significantly different.

Polydispersity index was unaffected by media or humic acid concentration, with a mean PDI of 0.49 (±0.01) across all test conditions (Table 2.3), signifying NM300K suspensions were halfway between fully uniformly dispersed (all particles of identical size and shape, PDI = 0) and fully non-uniformly dispersed (no particles of identical size and shape, PDI = 1).

2.6. Discussion

2.6.1. Silver dissolution

NM300K showed similar dissolution in all media without humic acid (i.e. unmodified OECD medium and JM, pH 6 and pH 8). In the absence of humic acid, there was a slight time-dependent increase (after 72 hours) in particle dissolution, however, the average dissolution was ca. 2-5% of total silver. This supports the hypothesis that NM300K dissolution will be < 10% in unmodified media. It was also demonstrated that dissolved silver was significantly higher in JM compared to OECD medium, supporting the view that media composition can influence nanoparticle dissolution. Significant increases in dissolved silver over time were observed most
media (except in OECD medium containing 5 mg/l SRHA, OECD medium buffered to pH 8, and unmodified JM. Also in JM buffered to pH 8 where dissolved silver significantly decreased). Published dissolution data for NM300K are limited, but range from < 0.01 – 0.78 % (Klein et al., 2011; Kermanizadeh et al., 2013), < 2% (Volker et al., 2013), 2-4 % (Mallèvre et al., 2014), and 1-10 % (Wang et al., 2012). Studies in which dissolved silver is measured have been conducted in a variety of media and at different timepoints, and used different approaches to quantify silver concentration which may account for differences in observed dissolution. Furthermore, methods of preparing samples for measuring dissolved silver may affect the results. AAS/ICP-MS are the most frequently used methods of measuring dissolved silver in NM300K studies, and require the nanoparticles to be removed from suspension (Klein et al., 2011; Kermanizadeh et al., 2013; Volker et al., 2013; Mallèvre et al., 2014). This is usually performed via either centrifugal ultrafiltration (Kermanizadeh et al., 2013; Volker et al., 2013) or by centrifugal sedimentation of nanoparticles (Mallèvre et al., 2014, and the work presented here). Some authors have questioned whether ultrafiltration units may retain some of the dissolved silver ions in nanoparticle suspensions and thereby underestimate dissolution (Gondikas et al., 2012; Gunsolus et al., 2015). This is particularly relevant to NM300K, as studies employing ultrafiltration often gave lower estimates of dissolution (Kermanizadeh et al., 2013; Volker et al., 2013) than those which did use other separation methods (Mallèvre et al., 2014; Wang et al., 2012; and the work presented here). Wang et al. (2012) used an ion selective silver electrode to obtain instantaneous measurements of NM300K dissolved silver, removing the need for sample preparation and later analysis. Studies of other silver nanoparticles have also used this method to increase sample frequency and describe kinetics of ionic silver release (Gondikas et al., 2012; Gunsolus et al., 2015). As such, this method may prove useful in future studies where in situ measurements of dissolved silver are desirable, giving better time-resolution and allowing measurements of toxicity test concentrations without the need to remove the test organisms.

A number of authors have found increased dissolution of silver nanoparticles with decreasing pH, which is enhanced in aerobic environments (Liu and Hurt, 2010; Oukarrum et al., 2014). In the ICP-MS data shown here for both unmodified media, dissolution of NM300K was significantly higher in JM compared to OECD medium at both 0 and 72 hours. This result may be attributable to the fact that the pH of JM is 0.8 units lower than OECD medium, when both media are in atmospheric equilibrium (Appendix 1). In addition, both media buffered to pH 6 exhibited significantly higher NM300K dissolution over time than either unbuffered media or media buffered to pH 8 (In JM, pH 8 medium actually resulted in a small but significant decrease in NM300K dissolution). The finding that silver nanoparticle time
dependent dissolution is enhanced at a lower pH aligns with studies in the published literature (Liu and Hurt, 2010). However the fact that significant differences in dissolved silver were observed between both media buffered to the same pH level indicates that, in the two test media investigated, other phenomena besides pH are be responsible for observed differences in nanoparticle dissolution such as ionic strength, medium composition, etc.

Dissolution of NM300K in the presence of humic acid was much higher in both media; an effect which was SRHA concentration and time dependent. That is, dissolution was greater at higher SRHA concentrations and 72 hours in both media. Liu and Hurt (2010) demonstrated that the addition of 50 mg/l SRHA reduced dissolved silver in a citrate-stabilised silver nanoparticle suspension [50 µg/l Ag] to almost zero over 24 hours. By contrast, other studies have found an increased 4 hour dissolution of citrate-stabilised silver nanoparticles in the presence of Leonardite humic acid (Pokhrel et al., 2013; 2014). However, Gao et al. (2012) found no significant effect of SRHA on bare silver nanoparticle dissolution after 5 days of incubation in deionised water. Studies of silver nanomaterial dissolution in the presence of humic acid generally suffer from a lack of standard methodology, the use of a variety of different silver nanoparticles and humic acid combinations. Methodological differences in sample preparation for characterisation include ultracentrifugation (Gao et al. 2012; Pokhrel et al., 2013; 2014), ultrafiltration (Liu and Hurt, 2010) and in situ ion selective electrode methods (Gunsolus et al., 2015), which may be attributable to differences in reported dissolution. For example, Gondikas et al. (2012) has attributed differences in published dissolution studies using ultrafiltration and ultracentrifugation methods to a loss of silver to the filtration units used in the latter. In addition, the expression of humic acid concentration as total mass (e.g. NOM) or as total/dissolved organic carbon makes comparisons between studies difficult. NOM describes a wide range of chemically heterogeneous mixtures, but many have been shown to have a high affinity for Ag⁺ (Janes and Playle, 1995; Bury et al., 2002; Mousavi et al., 2015). Compared to published literature the results presented here show unusually high dissolution (up to ca. 45 % in OECD medium containing 50 mg/l SRHA). Whilst humic acid reduced the pH of both media by around 0.5 - 0.7 units (measured at 0 hours in growth inhibition experiments – Chapter 3), such a change is not large enough to fully account for the increased dissolution compared to pH manipulation studies. To date, a detailed mechanistic understanding of silver nanoparticle and humic acid interaction has not been experimentally established, which makes understanding the cause of such high dissolution in this work problematic. If methodological issues indeed present a potential source of variation (Gondikas et al., 2012; Gunsolus et al., 2015), future work could include through cross validation of methods and the use of ionic controls of known dissolution.
Measurements of silver dissolution in newly opened, and one year old vials were analysed using ICP-MS. Dissolved silver in both vials was similar to that observed in the different media presented here, and to studies using similar methods of characterisation (Wang et al., 2012; Mallèvre et al., 2014). NM300K vials are packaged under an argon atmosphere to limit oxidative dissolution, and are recommended to be used immediately after opening (Klein et al., 2011). Despite this, dissolution between freshly opened and year old vials was not significantly different (3.75 %). The validity of this finding is very limited, as only two vials were used (many more were used in the course of this thesis), however these data tentatively suggest good inter-batch reliability in terms of dissolved silver content of NM300K. In future work it would be highly valuable to measure dissolved silver in every batch of NM300K, and in every toxicity test performed, but this was not possible due to the sample preparation/off-site ICP-MS measurement time and the cost and time associated with performing this work. As mentioned previously, ion selective silver electrodes may help accomplish this in future studies (Wang et al., 2012; Gondikas et al., 2012; Gunsolus et al., 2015). However for other characterisation data (e.g. measured using TEM, DLS, etc.), labour intensive sample preparation protocols (e.g. removal of test organisms, limits of detection, time and cost to measure each sample) are still limiting data to representative samples from test systems, rather than systematic characterisation data from every test performed.

Recovery (i.e. combined concentration of silver in supernatant and pellet) for the few samples tested were reasonable. Conclusions based on recovery data should be accepted only tentatively as data were only obtained from four unreplicated samples. With this in mind, it seems that longer duration in the test system results in poorer recovery. This was mostly caused by a reduction in the ability to detect/recover particulate silver, whilst dissolved silver remained similar to other test conditions. Such behaviour is consistent with the literature, and is attributed to sorption of both nano- and ionic silver to the test vessel (Malysheva et al., 2015; Lee et al., 2005), especially in the case of glass (Sekine et al., 2015). Such losses may have an influence in changes in toxicity over the standard OECD test duration and will be considered in Chapters 3 and 4.

2.6.2. Hydrodynamic diameter and zeta potential

In unmodified media and 18 mΩ water, NM300K hydrodynamic diameter was around 30 nm which aligns with the hydrodynamic diameter of NM300K particles observed in the published literature, in a variety of media/test systems/NM300K concentrations; such as bacterial (Losasso et al., 2014; Mallèvre et al., 2014; Donnellan et al., 2016), human cell culture (Johnston et al., 2015; Kermanizadeh et al., 2016) and environmental medium (Volker et al.,
For studies which cross validated DLS data using TEM, it was found that both methods showed similar estimates of size for NM300K (Losasso et al., 2014; Johnston et al., 2015; Donnellan et al., 2016; Kermanizadeh et al., 2016). Given the sensitivity of DLS measurements of particle hydrodynamic diameter to ionic strength (Klein et al., 2011; Malvern Instruments, 2013), such a result may be surprising. The DLS results estimate a lower mean hydrodynamic diameter than that stated by the JRC and some other authors (Klein et al., 2011; Sorensen and Baun, 2015), though this may be affected by factors such as NM300K concentration, dispersion protocols, and medium composition (e.g. factors not stated in Klein et al., 2011).

Humic acid moderately but significantly increased the hydrodynamic diameter of NM300K nanoparticles in JM, in a concentration dependent manner. A similar significant effect was observed in OECD medium, however NM300K hydrodynamic diameter was not as clearly affected by humic acid concentration (i.e. no significant differences in particle size were observed between 5 and 50 mg/l SRHA). Increased particle size may be due to associations between SRHA and the NM300K particle surface (Gunsolus et al., 2015). Many authors have demonstrated the stabilising effect of humic substances on silver nanoparticles prone to aggregation/agglomeration (e.g. Gunsolus et al., 2015; Gao et al., 2012; Delay et al., 2011). Previous studies demonstrate NM300K already exists in a well-stabilised state (Klein et al., 2011; Wang et al., 2012; Volker et al., 2013; Losasso et al., 2014; Mallèvre et al., 2014; Johnston et al., 2015; Donnellan et al., 2016; Kermanizadeh et al., 2016) and whilst the effect of humic acid on physical characteristics (hydrodynamic diameter, zeta potential, and polydispersity index) was sometimes significant, generally the impact on DLS measured characteristics appeared to be small.

The zeta potential measured in this study is also similar to published literature; negative but > -30mV (Wang et al., 2012; Volker et al., 2013; Sorensen and Baun, 2015; Donnellan et al., 2016). In an electrostatically stable suspension, particle zeta potential must be sufficiently high (< -30 or > 30 mV) to overcome weak attractive forces (Hanaor et al., 2012; Malvern Instruments, 2013). A low zeta potential, in absolute terms, was observed for NM300K, and the PDI was unaffected by the media conditions studied. Similar particle properties were found across a number of different characterisation studies (Klein et al., 2011; Volker et al., 2013; Mallèvre et al., 2014; Johnston et al., 2015; Sorensen and Baun, 2015; Donnellan et al., 2016; Kermanizadeh et al., 2016) which indicates NM300K exhibits high stability in a range of media. NM300K particles dispersed in OECD medium exhibited a significantly more negative zeta potential than in JM. The zeta potential of particles is related to the pH of the suspension; with higher pH (more alkalis) generally causing a larger negative charge, and lower pH (more acidic) a more positive (Ghosh et al., 2008; Peretyazhko et al.,

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As described previously, OECD medium has a higher pH (~7.8) than that of JM (~7.0) (Appendix 1), so the moderate increase in pH between OECD and JM may explain the significantly greater zeta potential observed in unmodified OECD medium.

Due to the presence of non-ionic surfactants polyoxyethylene (20) sorbitan mono-laurat and polyoxyethylene glycerol trioleate in NM300K dispersions, it is likely that steric stabilisation is the governing stabilising mechanism in this representative nanoparticle, as steric stabilisation is characterised by being relatively insensitive to differences in media, and nanoparticle concentration (Napper, 1982; Tauer, 2004). Whilst differences in electrostatic stability (as evidenced by zeta potential) may be noted, they may not confer any meaningful difference to overall system stability if steric stabilisation is the dominant mechanism of NM300K dispersion in aqueous media. Given the literature on both NM300K (Klein et al., 2011), and the properties of similar non-ionic polymer nanoparticle dispersions (Chappell et al., 2011; Tauer et al., 2004), the stability of this nanoparticle in a wide range of media may be assumed. However further work using orthogonal characterisation methods such as atomic force microscopy, nanoparticle tracking analysis or UV-vis spectroscopy could be used to cross-validate such assumptions in future work (Tomaszewska et al., 2013; Domingos et al., 2009).

The inclusion of humic acid in particle suspensions decreased the pH of both media. Thus, it could be assumed that this would decrease the zeta potential of particles suspended in humic acid containing medium. In fact, humic acid had little significant effect on the zeta potential of NM300K particles; except in JM containing 50 mg/l SRHA, where zeta potential was slightly but significantly more negative than either unmodified JM or JM containing 5 mg/l SRHA. The small humic acid associated increase in NM300K hydrodynamic diameter may indicate a shift in location of the shear plane, a boundary of closely associated charged molecules and/or ions around the particle. It is at this boundary at which zeta potential is measured (Malvern Instruments, 2013), and as a result the change in zeta potential may represent a change in location of measurement, rather than a change in value at a fixed location. The significant effect of humic acid on particle size in JM may further support this hypothesis, as a humically-induced increase in zeta potential was also observed in this medium. Delay et al. (2011) found the addition of NOM (in the form of natural bog water) increased silver nanoparticle hydrodynamic diameter (relative to 18 mΩ water), and maintained a negative zeta potential despite reducing pH, therefore leading to both a steric and electrostatic stabilisation effect. Furthermore, this effect was unaffected by salt concentration, which may explain the zeta potential convergence at 50 mg/l SRHA across the different media. This effect seemed to be SRHA concentration dependent in both media, which further supports this hypothesis.
Very few authors using DLS to study silver nanoparticles have presented PDI data, however such value is useful for identifying particle heterogeneity/stability in suspension (which may be increased by agglomeration/aggregation caused by media conditions/time). Authors who did investigate this measurement found similar results to those described here (Losasso et al., 2014; Mallèvre et al. 2014; Donnellan et al., 2016). Polydispersity index is a useful measurement of dispersion stability provided by DLS, but is rarely mentioned in DLS studies (Chappell et al., 2011). Having a PDI of ca. 0.5, indicates that NM300K particle suspensions are neither completely uniform (same size and shape; PDI = 0) nor completely non uniform (inconsistent size and shape; PDI = 1). This is likely due to the fact that NM300K is a nanoparticle suspension of a stated primary particle size of 15 nm, with a smaller proportion of 5 nm particles (Klein et al., 2013). DLS performs poorly on multimodal size range samples (Cho et al., 2013; Lee et al., 2013), so relying on this method for NM300K (i.e. which has a bimodal size distribution) may be unreliable. Multimethod approaches have been used to characterise nanoparticles in order to minimise the disadvantages of one particular method (Domingos et al., 2009). Despite this, characterisation at toxicologically-relevant concentrations across a broad variety of environmental samples poses many methodological challenges (Fabrega et al., 2011).

The observation that NM300K PDI was unaffected by any of the variables investigated (medium composition, humic acid addition) in this work indicates that the particles retain a similar size distribution in all media investigated. Slightly increasing hydrodynamic diameter as a result of SRHA addition, coupled with a stable PDI, indicates humic acid may form a coating around NM300K particles. As most published studies which have investigated the influence of humic acid on particle size have been conducted on highly aggregating/agglomerating silver nanoparticles (e.g. Fabrega et al., 2009; Gao et al., 2012; Badaway et al., 2010), NM300K may represent a unique example of the effect of humic acid on an already stable suspension.

Although used in toxicity tests, and characterised by ICP-MS, the effect of pH was not studied using DLS for a number of reasons. The most crucial of these is the limit of detection in DLS. Whilst NM300K concentrations of 5 and 10 mg/l Ag showed no significant difference in DLS results, these concentrations are up to 100 times higher than those used in the work carried out in standard OECD tests (Chapter 3), and higher still than current natural concentrations of silver in the environment (Luoma, 2008). Whilst future work could consider the effect of pH on NM300K, it is hypothesised (based on these findings and those of other studies) that if the associated dispersants cause the observed steric stabilisation in a range of media, pH is likely to have little effect on DLS derived measurements.
Transmission electron microscopy (TEM) was not used in this thesis, a choice made in favour of more detailed ICP-MS dissolution studies. TEM has been used to validate NM300K’s stated primary particle size (Klein et al., 2011), and this has found good concordance of results with other published studies which have investigated the morphology of size of NM300 nanoparticles in biological medium using TEM (Kermanizadeh et al., 2013; Losasso et al., 2014; Donnellan et al., 2016). Furthermore, detailed TEM investigation of the size and morphology of NM300K, in a range of test conditions, has been performed by colleagues at Heriot Watt University (unpublished observations; Figures 2.8-2.10, and Klein et al., 2011; Figure 2.11). TEM does not reveal a great deal of qualitative difference between NM300/NM300K particles dispersed in different media (Figures 2.8-2.11). Particles are typically well dispersed, with limited evidence of particle agglomeration/aggregation. 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 particles observed in different studies. Given these results, TEM was not carried out in this thesis due to the apparent stability of NM300K in a variety of media. In addition, TEM has more 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 as nanoparticles, or false-agglomeration during evaporation (Pettitt and Lead, 2008; Domingos et al., 2009).

Figure 2.8: TEM image of NM300K in 18 mΩ water, provided by Samutrtai (2016). NM300K particle concentration 2 mg/l (left) and 5 mg/l (right), timepoint = 24 hours.
Figure 2.9: TEM image (Philips CM-200 FEG) of NM300K at 26500x magnification, in 18 mΩ water (left) and OECD 315 *Lumbriculus variegatus* medium (right), provided by Little (2016). NM300K particle concentration = 10 mg/l, timepoint = 2 hours.

Figure 2.10: TEM image of NM300K in Middlebrooks 7H9 broth, provided by Donnellan (2016). NM300K particle concentration 25 mg/l (left) and 100 mg/l (right), timepoint = 0 hours.
Finally, all characterisation was carried out in pristine, algae free media. Whilst such data are important, algae can modify media conditions through changes in pH (Auroja, 2011) and through the production of poorly quantified “exopolymeric substances” (Koukal et al., 2007) which may further influence metal speciation, nanoparticle properties, exposure conditions, and ultimately toxicity. As a result, further studies should move towards a better understanding of how such processes affect chemical speciation over the duration of a standard OECD test.

2.7. Conclusions

The results presented here indicate that NM300K silver nanoparticles exhibit good stability in a range of aqueous media, which agrees with the findings of other published studies. This is most likely due to the properties conferred by the two non-ionic polymeric stabilising agents (polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan monolaurat (Tween 20)). Of benefit is that as the nanoparticles tested in this study were obtained from the JRC nanomaterial repository the physico-chemical properties of the pristine nanoparticles (e.g. size, morphology, surface area) have been extensively characterised. Thus, these published data can be used in conjunction with the data obtained in this study to help identify what properties of NM300K particles may confer toxicity.

The choice of which characterisation methods to deploy may vary depending on the nanomaterial under investigation, the suspected mechanism of toxicity, and test system as well as financial and time considerations. DLS was chosen so that the data obtained could be
compared to existing studies which have characterised NM300K in different media. Furthermore a detailed investigation of silver dissolution was performed, in conditions representative of toxicity work with *R. subcapitata* (media, NM300K concentration, incubation time). Dissolution was specifically chosen for more detailed investigation due to the uncertainty over the dominant toxic effect of silver nanoparticles to algae (i.e. ionic or nanoparticles).

These results contribute additional knowledge to the dissolution of NM300K in aqueous media. With current uncertainty over the dominant toxic effect of silver nanoparticles (free ions vs. nanoparticle), such dissolution studies are crucial to understanding the relative importance of ionic silver in such toxicity tests. Whilst SRHA appears to significantly promote NM300K dissolution, assuming this is not an artefact of sample preparation, this does not necessarily mean that the increased silver is bioavailable to cause toxic effects (Chapter 3).
Chapter Three - Silver toxicity in standard tests

3.1. Introduction

Metal nanomaterials, being of identical chemical composition to their ionic (for metals that readily dissolve) and bulk equivalents, are not yet designated by regulatory bodies as separate entities requiring their own specific regulation. As such, there is considerable interest in identifying, monitoring and refining the suitability of OECD test guidelines for the purposes of regulating nanomaterials due to their novel physicochemical properties which may not be adequately provided for in some standard test systems (Handy et al., 2008).

3.1.1. OECD test background

OECD Guidelines for the Testing of Chemicals were first developed in 1981. They provide a specific set of internationally-accepted protocols designed to assess the hazard of chemicals to both human health and the wider natural environment, across five sections, as described below (OECD, 2016):

1. Physical and Chemical Properties: tests assessing properties such as solubility, adsorption, and particle size help to determine exposure.
2. Effects on Biotic Systems: tests employing a variety of animal, plant and bacterial models to quantify environmental hazard.
3. Degradation and Accumulation: tests quantifying biotic and abiotic transformation processes to determine environmental persistence.
4. Health Effects: primarily tests on human analogues (cell lines and animal models) to determine specific hazard to a variety of physiological processes.
5. Other Tests Guidelines: mostly tests relating to agriculture such as crops, livestock and pesticide persistence.

Test guidelines are covered by the Mutual Acceptance of Data (MAD); data gathered using the guidelines, by OECD member or partner countries, are freely shared and accepted between all OECD members and partners for the benefit of both human health and the environment. The application of OECD test guidelines is used for regulatory purposes, and to ensure wider applicability and comparison across laboratories (OECD, 2016).

The regulatory requirement for comparable results between tests conducted in different laboratories has been the focus of discussions in regards to their low environmental
relevancy, and thus poor coverage of the natural environment (Chapman, 2002). For example, very few marine organisms are included in Section 2 of the OECD test guidelines (see above), with the exception of a few fish and microalgal species. In reality, test organisms are often selected based on their ease of husbandry and sensitivity to a range of substances under laboratory conditions, rather than applicability to the environment in which particular toxicants may be commonly encountered (Chapman, 2002). Laboratory studies are typically carried out in well-defined artificial media which can easily be manipulated to account for toxicant specific phenomena such as chelation (OECD, 2011). Developing complex, multispecies mesocosm experiments is outside the scope of this thesis, however a preliminary level of additional environmental complexity has been included, in the form of humic acid (Suwannee River humic acid - SRHA) and by modifying pH. Such modifications are not stipulated in the standard test protocol (OECD, 2011), but may serve to enhance the understanding of silver nanomaterials toxicity in natural environments.

3.1.2. Toxicity of silver to algae

It is important to situate the data gathered in this chapter within current understanding of ionic silver uptake and toxicity in algal species. As silver has no biological role, Ag⁺ is hypothesised to enter algal cells incidentally though cationic Cu(I) transporters (Lee et al., 2004). Silver shares a number of physico-chemical properties with copper (both group IB transition metals), and has been found to be internalised through Cu(I) transporters in bacteria (Solioz and Odermatt, 1995; Rensing et al., 2000), cyanobacteria (Phung et al., 1994), fungi (Riggle and Kumamoto, 2000) and human kidney cells (Lee et al., 2002). Furthermore, Enterococcus hirae cells, which had been genetically altered to not express Cu(I) uptake transporters, showed increased resistance to Ag(I) toxicity, which is likely to derive from reduced Ag uptake by cells (Odermatt et al., 1994). Although the existence of similar Cu(I) transport proteins have not been described for R. subcapitata, parallel discoveries across a range of taxonomic groups, coupled with the biological role of copper (Festa and Thiele, 2011), suggest the ubiquitous existence of such transporters (Lee et al., 2004).

The Biotic Ligand Model (BLM) is commonly used to describe interactions between metals and organisms, and assumes soluble metal speciation close to an organism’s cell membrane will interact with the cell, and ultimately result in uptake (i.e. become bioavailable) and cause concentration-dependent toxicity (Morel, 1983). This model relies on a number of assumptions (Campbell et al., 2002; Lee et al., 2004):
1. Metal flux towards the cell membrane and resulting complexation occurs more rapidly than the rate of uptake by the cell (i.e. diffusion towards the cell is not rate-limiting).

2. The cell membrane is the primary metal interaction location, and metal uptake is mediated through an active mechanism.

3. Uptake and toxicity are directly dependent on cell membrane concentration of the complexed metal.

4. Available cell membrane transport/complexation sites are sufficient to not limit metal availability.

5. The cell membrane remains unchanged by the metal.

In the case of ionic silver (Ag⁺) and algae, BLM is thought to be applicable, but only in algae which exhibit “slow” uptake rates such as *R. subcapitata* and *Chlorella pyrenoidosa* (Lee *et al.*, 2004; 2005). In these species, Ag⁺ uptake rates are thought to be low enough to avoid Ag⁺ diffusion to the algal cell surface exerting rate-limiting effects (Lee *et al.*, 2004; 2005).

Ionic silver uptake in algae is enhanced in the presence of thiosulphate and other sulphurous (II) ligands, which have a high affinity for silver (logK ≥ 8.2) (Fortin and Campbell, 2001; Kramer *et al.*, 2002; Hiriart-Baer *et al.*, 2006). These complexes are thought to enter the cell via a separate pathway to Ag⁺, through one or more anionic sulphate/thiosulphate transporters (Fortin and Campbell, 2001). Transport via this mechanism would limit the applicability of BLM for silver nanoparticles in algae as uptake is no longer solely determined on an Ag⁺ basis (Hiriart-Baer *et al.*, 2006). In the absence of reduced sulphurous compounds, silver has a weaker affinity for inorganic ligands – namely chloride (logK ≈ 3). This chloride complexation has the opposite effect to thiosulphate, where reduced *R. subcapitata* silver uptake and toxicity have been observed in medium containing 354.53 mg/l Cl, relative to the same medium containing 141.8 mg/l Cl (Lee *et al.*, 2004).

Silver nanomaterials are generally less toxic to both algae and other models, on a total metal basis than ionic silver (Bondarenko *et al.*, 2013; Table 3.16). The debate regarding to what extent silver nanomaterial toxicity to algae can be completely explained on the basis of dissolved Ag⁺ has been addressed in numerous studies (Navarro *et al.*, 2008; Miao *et al.*, 2009; Wang *et al.*, 2012; Gao *et al.*, 2012; Xiu *et al.*, 2012; Oukarroum *et al.*, 2013; Sorensen and Baun, 2015; Gunsolus *et al.*, 2015), although this research question has not been answered conclusively. A lack of standardised methodology between studies may result in errors in
adequately separating dissolved silver fractions from nanomaterials, and so modelling chemical speciation offers a potential *in silico* alternative to assess silver speciation in solution. Conversely, such models may calculate speciation in a “perfect” solution, that is one which is unaffected by the reality of potential losses of silver to the test system (Lee *et al.*, 2005; Malsyheva *et al.*, 2015; Sekine *et al.*, 2015). In reality such issues are difficult to address without *in situ* methods of quantifying Ag⁺ (Gunsolus *et al.*, 2015; Mousavi *et al.*, 2015).

### 3.1.3. The influence of pH on the toxicity of chemicals to algal species

Algal cultures naturally increase the pH of their environment, if CO₂ diffusion into the growth medium is inadequate (Arensberg *et al.*, 1995; Aruoja, 2011). OECD 201 test guideline allows for a pH excursion of < 1.5 units over the test duration (OECD, 2011); as pH is measured on a logarithmic scale, such a change is non-trivial and may have a considerable effect on toxicity (Rendal *et al.*, 2012). In addition to algal induced changes in pH, variations in pH are obvious phenomena in natural systems, so investigating the impact of pH on the ecotoxicity of nanoparticles is an important consideration when performing hazard assessments. A number of studies exist showing the reduction in algal toxicity with increasing pH (4.5-8) for metal ions such as copper, zinc, cadmium and nickel (Macfie *et al.*, 1994; Nalewajko *et al.*, 1997; Franklin *et al.*, 2000; De Schamphelaere *et al.*, 2004), but data on silver (nanomaterials or ions) toxicity to algae as a result of pH are limited (Oukarroum *et al.*, 2014). Oukarrum *et al.* (2014) studied the influence of a pH range of 4 to 7 on silver nanoparticle toxicity, and as a result had to use an acidophilic algal species (*Chlamydomonas acidophila*). These authors found that acidic pH increased the dissolution of silver nanoparticles, which in turn increased toxicity as indicated by reduced cell viability and increased reactive oxygen species (ROS) production. In order to determine the effect of pH on silver toxicity in *R. subcapitata*, which has a pH tolerance of 6.5-9 (Environment Agency, 2008), a narrower pH range must be considered. At a pH of 6.5 or 8, Seitz *et al.* (2015) found that higher pH was associated with reduced toxicity (indicated by 48 hour mortality and 21 day reproduction) of both silver nitrate and silver nanoparticles to *Daphnia magna*. As described in Section 1.3, the dissolution of silver nanomaterials requires both oxygen and protons (Liu and Hurt, 2010; Xiu *et al.*, 2012). Therefore for a given test system, replete in oxygen, the concentration of protons (i.e. pH) will be the rate limiting factor to silver nanomaterial dissolution. It follows that a lower pH will likely result in higher silver nanomaterial dissolution and so higher toxicity to algae. In addition, the effect of pH may have an indirect synergistic effect on silver toxicity through modifying the carbon availability and, as a result, algal growth (Falkowski and Raven, 2007)
OECD guideline 201 does not require an investigation of the influence of pH on toxicity to algae (OECD, 2016). Furthermore, no studies were identified that investigated the influence of pH on silver nanoparticle toxicity to *R. subcapitata*. Therefore consideration of the effects of pH on silver (ionic and nanoparticle) toxicity was considered relevant to adequate regulatory assessment using this OECD test guideline.

Due to algal modification of pH over their growth period, the use of a buffering system is necessary when studying the influence of a fixed pH on chemical toxicity. OECD medium has an intrinsic buffering capacity due to the presence of NH$_4^+$ as the primary nitrogen source, which offsets algal induced increases in pH through the liberation of protons (Arensberg *et al.*, 1995; Aruoja, 2011). However OECD medium’s buffering capacity is only effective at the test system pH of ~7.0 (measured from toxicant free cultures of *R. subcapitata*), so additional buffering is required to study other pH levels. The choice of buffer in ecotoxicology tests is ultimately the result of balancing a range of competing practical considerations such as: toxicity to test species, target pH (and acceptable drift), potential complexation with toxicant/medium components, and environmental relevancy (Good *et al.*, 1966; Rendal *et al.*, 2012; Mousavi *et al.*, 2015). Whilst some authors, desiring very strict pH control, have identified suitable buffers (e.g. 2-amino-2-hydroxymethyl-propane-1,3-diol, also known as Tris buffer) for use in algal tests (Rendal *et al.*, 2012), OECD test guideline 201 allows a pH change of < 1.5 units which can adequately be achieved with a range of buffers (OECD, 2011; Rendal *et al.*, 2012). MOPS (3-(N-morpholino)propanesulfonic acid) was chosen in this study due to its low toxicity, adequate buffering according to OECD test guidelines, and high solubility (Good *et al.*, 1966; De Schamphelaere *et al.*, 2002; Rendal *et al.*, 2010). In addition it has been successfully used in other studies of algal toxicity (De Schamphelaere *et al.*, 2002; Navarro *et al.*, 2008; Chen *et al.*, 2013).

### 3.1.4. The influence of humic acid on the toxicity of chemical substances to algal species

Water chemistry in natural environmental systems is highly complex, with a great deal of spatial heterogeneity. Natural organic matter (NOM) is found commonly throughout aquatic systems. NOM is a broad mixture of organic compounds, which originate from both aquatic and terrestrial sources. Terrestrial sources are derived from soil run off and are often characterised by low molecular weight (< 1 kDa) compounds produced by soil microbes and higher plants. Aquatic sources include remineralised, high molecular weight (> 1 kDa) polysaccharides and peptidoglycans from phytoplankton and macrophytes (Wilkinson *et al.*, 1997).
Collectively a large part of NOM in freshwater systems is as classified humic substances, a group of organically derived compounds considered to be resistant to degradation by bacteria (Baker, 1991). The typical chemical composition of humic substances is \(~50\%\) carbon, 4-5 \% hydrogen, 35-40 \% oxygen, 1-2 \% nitrogen, and < 1 \% sulphur (Baker, 1991). Streams, rivers and lakes contain between 0.5-5 mg/l C, in the form of humic substances. Dystrophic waters, characterised by their low nitrogen and high acidity (e.g. marshes and swamps), contain up to 30 mg/l C as humic substances (Rand et al., 1995). Due to their complex nature, humic substances can have a significant effect on the bioavailability of toxic metals and other chemicals, through mechanisms such as chelation, flocculation, dispersion, and sedimentation (Rand et al., 1995). Humic substances have been shown to stabilise and disaggregate metal colloids (Tipping and Higgins, 1982; Wilkinson et al., 1997), as well as nanomaterial suspensions (Baalousha, 2009; Gao et al., 2012, Omar et al., 2014), though this effect is highly dependent on particle type and media properties. In addition, humic acid can also enhance iron bioavailability resulting in increased algal growth (Kean et al., 2015).

Several studies have found that humic substances reduce the toxicity of silver nanomaterials to a number of model organisms including bacteria (Fabrega et al., 2009; Wirth et al., 2012; Zhang et al., 2012; Gunsolus et al., 2016), daphnids (Gao et al., 2012), Caenorhabditis elegans (Yang et al., 2014) and Oryzias latipes embryos (Kim et al., 2013). The ability of humic substances to mitigate silver’s toxicity has been attributed to their affinity for silver, which may both increase nanomaterial agglomeration/sedimentation (Gao et al. 2012) and remove bioavailable silver ions from the test medium (Liu and Hurt, 2010). Despite this, Chen et al. (2013) found enhanced ionic silver uptake by R. subcapitata and Chlamydomonas reinhardtii in the presence of Suwannee River humic acid, after 25 and 60 minutes of exposure respectively. The authors performed a more comprehensive assessment of subcellular silver distribution and toxicity using C. reinhardtii, and demonstrated that silver was predominantly distributed in organelles (30-40 \% total cellular Ag). Even though patterns of subcellular silver distribution in C. reinhardtii broadly applied to both media with and without humic acid (5 and 10 mg/l C, as SRHA), SRHA slightly but significantly enhanced uptake associated with the cell surface (around 5-10 \% of relative silver distribution). This effect was hypothesised to be driven by binding of silver-humic acid complexes at the surface of the cell, and had no effect on ionic silver toxicity (24 hour growth inhibition) in this species (Chen et al., 2013). The toxicity mitigating effect of humic substances may depend on the concentration of both silver and humic acids, on both a relative and absolute basis. A humic acid, concentration-dependent reduction in silver toxicity has been observed by a number of authors (Fabrega et al., 2009;
Gao et al., 2012; Kim et al., 2013). In addition, not all humic substances exert a similar effect on silver; those containing more nitrogen and sulphur tend to show a higher degree of free silver complexation and resulting reduction in toxicity (Mousavi et al., 2015). OECD guidelines do not stipulate the use of humic substances in chemical hazard assessments, however the importance of understanding their effect on toxicity in naturalised settings should not be understated. Laboratory studies using different natural water samples (containing environmentally-relevant amounts of humic substances), indicate that natural waters containing a greater amount of humic substances can exert higher mitigating effects on silver nanomaterial toxicity (McLaughlin and Bonzongo, 2012; Zhang et al., 2012).

3.1.5. The impact of growth media composition on algal toxicity

OECD test guideline 201 recommends the use of two different algal growth media (US Environmental Protection Agency AAP medium and OECD 201 medium), which differ slightly in their initial pH, buffering capacity and chemical composition (OECD, 2011). Other test media are permitted on the provision that their use is methodologically justified. A number of studies, using both standard and adapted OECD 201 test designs, have used non standard media to assess toxicity of various substances in R. subcapitata. These include Bristol medium (Johnson et al., 2007; Labra et al., 2007) and Jaworsky’s medium (Salomao et al., 2014).

Some studies have considered how the toxicity of silver is modified in algal growth inhibition tests, as the result of changing one aspect of the growth medium. These include Cl⁻ (Lee et al., 2004; 2005), pH (Macfie et al., 1994; Nalewajko et al., 1997; Franklin et al., 2000; De Schamphelaere et al., 2004), and humic substances (Fabrega et al., 2009; Gao et al., 2012; McLaughlin and Bonzongo, 2012; Zhang et al., 2012; Chen et al., 2013; Kim et al., 2013; Mousavi et al., 2015). Despite permitting different media compositions in the OECD 201 test (OECD, 2011), very little regulatory attention has been given to the possibility of differences in toxicity being observed between such media. Such differences would need to be taken into account when comparing results between different studies.

A non-standard medium (Jaworsky’s medium - JM), as well as the standard OECD medium, were chosen to investigate silver toxicity to algae in this chapter. Compared to OECD medium, JM contains more EDTA, iron and has a higher ionic strength (Appendix 1). Iron is an essential element for algal growth but requires EDTA to remain in solution (Kean et al., 2015), so growth rate may be increased in JM if iron is rate limiting in OECD medium. This may have implications on relative growth inhibition calculations when compared to OECD medium. Higher EDTA concentrations have been demonstrated to enhance silver nanomaterial dissolution and dispersion (Chappell et al., 2011), however small amounts of EDTA (i.e.
sufficient amounts to chelate trace elements, and not in excess) in the test medium are preferable to potential poorly understood natural chelating agents which algae may secrete in order to aid absorption of trace metals (Aruoja, 2011; OECD, 2011). Increased ionic strength (in the form of seawater) has been shown to reduce silver ion release from particles (Liu and Hurt, 2010), however the difference in ionic strength observed between OECD medium (1.67 mmol/l) and JM (4.08 mmol/l) does not support this finding (Figure 2.1; higher NM300K dissolution in JM). In addition, JM lacks the buffering capacity of OECD medium due to NO$_3^-$ being the primary nitrogen source (i.e. unlike NH$_4^+$ in OECD), which may have implications on test system pH and resultant toxicity as discussed above. Finally, chloride concentration in OECD medium (22.99 mg/l) is around 4.6 times higher than JM (0.5 mg/l). As chloride has been determined to reduce toxicity of silver to algae (Lee et al., 2004), this difference may have an effect on observed toxicity in these media.

### 3.2. Aims and objectives

The aims of this chapter were as follows:

- To investigate the impact on ionic and nanoparticulate silver (NM300K) on algal (*R. subcapitata*) growth (over a range of concentrations) using the standard OECD 201 algal growth inhibition test system.

- To investigate the influence of growth media selection (OECD and JM) on ionic and nanoparticulate silver toxicity to algae (*R. subcapitata*) using the OECD 201 algal growth inhibition test system.

- To assess the effect of pH (6 or 8) on silver (both ionic and nanoparticle) toxicity to algae in both OECD medium and JM. MOPS buffer and NaOH/HCl were used to achieve a pH of 6 and 8 in each growth medium.

- To assess the effect of humic acid (5 and 50 mg/l SRHA) on silver (both ionic and nanoparticle) toxicity to algae, in both OECD medium and JM.
3.3. Hypotheses

3.3.1. Research Hypotheses

- Silver nanoparticles will exhibit lower toxicity than ionic silver, calculated on a total metal concentration basis.

- Differences in silver (ionic and nanoparticle) toxicity between media will be primarily driven by differences in predicted ionic silver speciation.

- Silver (ionic and nanoparticle) toxicity will be lower in medium containing SRHA (5 and 50 mg/l). This toxicity mitigating effect will increase with increasing SRHA concentration.

- Silver (ionic and nanoparticle) toxicity will be lower in medium buffered to a pH of 8, compared to a pH of 6, due to a lower concentration of protons at higher pH levels.

3.3.2. Null Hypotheses

- There will be no difference in toxicity between nanoparticulate and ionic silver, calculated on a total metal concentration basis.

- There will be no difference in silver toxicity (nanoparticulate and ionic) between the two media, calculated on a total metal concentration basis.

- Humic acid concentration will have no effect on silver toxicity (nanoparticulate and ionic), calculated on a total metal concentration basis.

- Changing pH will have no effect on silver toxicity (nanoparticulate and ionic), calculated on a total metal concentration basis.

3.4. Methods

3.4.1. Glassware and toxicant stocks

Glassware for toxicant exposures was prepared as described in Section 2.4.1. Vials of silver nanoparticles suspended in a dispersant (NM300K; 10.16 % w/w silver) and dispersant only (NM300K-DIS; 4 % w/w% each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan mono-laurat (Tween 20)) were provided by the JRC. Silver nitrate (AgNO₃) was obtained from Fisher Scientific. All stock suspensions/solutions were prepared on the same day of testing.
Stocks of silver nanoparticles and AgNO₃ were made to a concentration of 100 mg/l Ag (w/v), by weighing 49.2 mg of NM300K and 7.87 mg of AgNO₃ into 50 ml 18 mΩ water respectively. NM300K-DIS was prepared to the same equivalent particle free concentration as NM300K, by weighing 44.2 mg of the dispersant into 50 ml 18 mΩ water. Toxicant stocks were sonicated and serially diluted as described in Section 2.4.1.

3.4.2. Algal stock cultures

*R. subcapitata* (Strain 278/4) was provided by CCAP (The Culture Collection of Algae and Protozoa, Oban, Scotland). During routine husbandry, stock cultures were continuously maintained in the CCAP recommended Jaworsky's Medium (JM) in 100 ml volumes in a sterile 250 ml borosilicate Erlenmeyer flask, sealed with a polyurethane foam bung; in static conditions, under 16:8 hour light:dark, ~50 µmol/m²/s, at 20°C ±2°C. Subculturing was carried out weekly.

Cultures were checked for contamination every two weeks by plating onto JM agar at 20°C ± 2°C. When necessary, cultures were purified by streaking onto JM agar and transferring isolated colonies into fresh medium.

Prior to use in a test, a sterile 250 ml borosilicate flask containing 100ml of freshly prepared medium (OECD medium or JM) was inoculated with a suitable algal culture volume (1-5ml, depending on the period of culture and intended incubation period), and placed in an orbital shaker (Multitron Standard, Infors-HT), at 225RPM, under continuous fluorescent light ~120 µmol/m²/s, at 23°C ± 2°C. Typical incubation periods ranged from 2-4 days depending on growth and inoculum density. Culture density was assessed prior to experiments at 685nm in a 10 cm quartz cuvette, using a dual beam UV/visible spectrophotometer (Jenway model 6715), and cultures in exponential phase (growth rate > 1.4 d) were used in experiments.

3.4.3. Impact of silver on algal growth

Standard OECD test exposures were carried out in accordance with OECD 201 Freshwater Alga and Cyanobacteria, Growth Inhibition Test (OECD, 2011). Toxicant test concentration volumes were made in 160 ml; this comprised of 40 ml of the test substance (NM300K, AgNO₃) at four times the desired test concentration in 18 mΩ water, 40 ml double strength medium (OECD medium/JM), and 80 ml exponentially growing R. subcapitata diluted to 1 x 10⁵ cells/ml. For controls, test volumes were made in 320 ml; this comprised of 80 ml toxicant free 18 mΩ water, 80 ml double strength medium (OECD medium/JM) and 160 ml exponentially growing R. subcapitata diluted to 1 x 10⁵ cells/ml. All test conditions were mixed in the order – base medium, followed by toxicant (or 18 mΩ water for controls), followed by
algal culture. The test volumes (toxicant-free controls and toxicant exposures) were then separated into 250 ml borosilicate Erlenmeyer flasks (~50 ml per flask), to prepare six replicate controls and three replicates of each toxicant concentration. After initial concentration range finding studies had been performed, silver toxicity to *R. subcapitata* growth was found to vary between both silver form (ions and nanoparticles) and medium. Test concentrations of ionic silver were 0, 0.2, 0.6, 2, 6, 20 µg/l Ag for OECD medium and 0, 1, 2, 7.5, 15, 30 µg/l Ag for JM. For nanoparticles, test concentrations were 0, 30, 60, 90, 120, 150 µg/l Ag for OECD medium and 0, 20, 30, 40, 50, 60 µg/l Ag for JM. Control studies of dispersant (NM300K-DIS) toxicity were carried out at the highest equivalent particle free NM300K concentration in both media (i.e. 150 µg/l in OECD and 60 µg/l in JM).

To determine algal cell density for growth inhibition tests, a simplified, Chl$_a$ extraction was performed on unfiltered algal samples, based on methods adapted from Mayer *et al.* (1997). In brief, a sample of the algal culture (1 ml) was taken at 24, 48 and 72 hours and placed in a 10 ml screw-cap glass tube. Locust bean gum solution (1.5 g/l locust gum powder in 18 mΩ water, heated to 50°C to aid dispersion) was then added (0.1 ml) to aid in the precipitation of particulates, and 4.4 ml of 100 % acetone (final acetone concentration in samples = 80 %) added to extract Chl$_a$ pigment. This solution was briefly manually shaken and placed for 3 days in the dark at 20°C. The supernatant was removed with a Pasteur pipette, transferred to a fluorometer cuvette and the concentration of chlorophyll was read in a calibrated fluorometer (Trilogy, Turner Designs) using the Chl-NA module. Solid state standards were used to check instrument drift periodically, and the instrument adjusted, if required.

Growth rate was calculated based on extracted Chl$_a$ concentration for each treatment replicate (including the control) at each time point using the following equation:

$$ \mu_{i,j} = ((\ln X_j - \ln X_i)/(t_j-t_i))$$

Where: $\mu_{i,j} =$ specific growth rate from time i to time j; $X_i =$ Chl$_a$ concentration at time i; $X_j =$ Chl$_a$ concentration at time j.

Growth inhibition was calculated for each individual treatment replicate, relative to the mean growth rate of the 6 controls, using the following equation:

$$%Ir = ((\mu c - \mu t)/ \mu c) \times 100$$

Where: $%Ir =$ percent growth inhibition in average specific growth rate determined from Chl$_a$ concentration; $\mu c =$ mean value for average specific growth rate ($\mu$) in the control group; $\mu t =$ average specific growth rate for the treatment replicate.
Experiments were run over a 72 hour period and measurements taken at 24 hour intervals. Experiments were repeated 3 times, with 3 replicates at each time.

3.4.4. The effect of pH and humic acid on silver toxicity to algae

A single concentration of ionic and nanoparticle silver was identified to test in humic acid and pH studies based on the results obtained from the concentration-response experiments. In order to study the effect of these media modifications on a fixed inhibition level of silver toxicity (both ionic and nanoparticulate), a silver concentration that was predicted to induce 80 % growth inhibition over 72 hours was calculated from probit modelling of previous concentration-response studies. In OECD medium this was determined to be 190 µg/l Ag for silver nanoparticles and 6 µg/l Ag for ionic silver. In JM this was determined to be 80 µg/l Ag for silver nanoparticles and 11 µg/l Ag for ionic silver. Two pH levels (pH 6 and 8; MOPS buffer) and two concentrations of Suwannee River humic acid (SRHA – 5 mg/l and 50 mg/l) were investigated. MOPS buffer was chosen for use in pH studies as it is non-complexing with metals (Kandegedara and Rorabacher, 1999), and has been successfully used in other toxicity studies with *R. subcapitata* (De Schamphelaere *et al*., 2002; Heijerick *et al*., 2002; Van Hoecke *et al*., 2008). The pH range was chosen on the basis of MOPS effective buffering range (pH 6-8), which is within the natural pH range of most freshwaters (Talling, 2010). SRHA used in this thesis contains 52.63 % carbon, 4.28 % hydrogen, 42.04 % oxygen, 1.17 % nitrogen, 0.54 % sulphur and 0.01 % phosphorus (International Humic Substances Society, 2016). Concentrations used are within the typical range of surface and groundwater humic acid concentrations in natural systems (0-50 mg/l - Li *et al*., 2009).

To modify both media for pH experiments MOPS buffer (C<sub>7</sub>H<sub>15</sub>NO<sub>4</sub>S), HCl and NaOH were used. MOPS stock solutions [0.5 M] were prepared in 18 mΩ water, and adjusted to pH 6 and 8, respectively, using NaOH, syringe filtered (pore size - 0.2 µm) and transferred into sterile acid-washed glass bottles.

The impact of pH on the toxicity of silver (ionic and nanoparticle) on algae was assessed using 6 test conditions for each media (made in 160 ml, or 320 ml for unmodified toxicant free controls, and separated into three ~50 ml replicates as in Section 3.4.3.) as follows. Unmodified toxicant free medium controls (6 replicates) comprised of 80 ml toxicant free 18 mΩ water, 80 ml double strength medium (OECD medium/JM) and 160 ml exponentially growing *R. subcapitata* diluted to 1 x 10<sup>5</sup> cells/ml. pH 6 (3.5 mM MOPS) toxicant free medium control (3 replicates) comprised of 2.8 ml MOPS stock [0.5 M] at pH 6, 20 ml toxicant free 18 mΩ water, 40 ml double strength medium (OECD medium/JM). This solution was adjusted to pH 6 using HCl (0.01 and 0.1 M) and topped up to a total volume of 80 ml,
before adding 80 ml exponentially growing *R. subcapitata* diluted to $1 \times 10^5$ cells/ml. pH 8 (3.5 mM MOPS) toxicant free medium control (3 replicates) comprised of 2.8 ml MOPS stock [0.5 M] at pH 8, 20 ml toxicant free 18 mΩ water, 40 ml double strength medium (OECD medium/JM). This solution was adjusted to pH 6 using NaOH (0.01 and 0.1 M) and topped up to a total volume of 80 ml, before adding 80 ml exponentially growing *R. subcapitata* diluted to $1 \times 10^5$ cells/ml. Silver at 80 % effective concentration (EC$_{80}$) (3 replicates) comprised of 40 ml of the test substance (NM300K, AgNO$_3$) at four times the desired test concentration in 18 mΩ water, 40 ml double strength medium (OECD medium/JM), and 80 ml exponentially growing *R. subcapitata* diluted to $1 \times 10^5$ cells/ml. Silver at 80 % effective concentration (EC$_{80}$) at pH 6 (3.5 mM MOPS) (3 replicates) comprised of 2.8 ml MOPS stock [0.5 M] at pH 6 and 40 ml double strength medium (OECD medium/JM). This solution was adjusted to pH 6 using HCl (0.01 and 0.1 M) and topped up to a total volume of 60 ml, before adding 20 ml of the test substance (NM300K, AgNO$_3$) at eight times the desired test concentration in 18 mΩ water, followed by 80 ml exponentially growing *R. subcapitata* diluted to $1 \times 10^5$ cells/ml. Silver at 80 % effective concentration (EC$_{80}$) at pH 8 (3.5 mM MOPS) (3 replicates) comprised of 2.8 ml MOPS stock [0.5 M] at pH 8 and 40 ml double strength medium (OECD medium/JM). This solution was adjusted to pH 8 using NaOH (0.01 and 0.1 M) and topped up to a total volume of 60 ml, before adding 20 ml of the test substance (NM300K, AgNO$_3$) at eight times the desired test concentration in 18 mΩ water, followed by 80 ml exponentially growing *R. subcapitata* diluted to $1 \times 10^5$ cells/ml. All test conditions were mixed in the order – base medium (with or without MOPS), followed by pH adjustment (HCl or NaOH), followed by toxicant (or 18 mΩ water for controls), followed by algal culture.

The volumes of NaOH and HCl added were recorded for each test condition; pH was measured using a probe (Orion ROSS Ultra, Thermo Scientific) connected to an electrochemistry meter (Orion Star Plus, Thermo Scientific) which was calibrated on the day of use. pH and electrical conductivity were measured daily in each flask for the duration of the experiment, using a pH probe (Orion ROSS Ultra, ThermoScientific) and a conductivity probe (Orion 4-cell, Thermo Scientific), connected to an electrochemistry meter (Orion Star Plus, Thermo Scientific). The test duration was 72 hours, following the same OECD protocol as described in Section 3.4.3.

In humic acid studies SRHA stock solutions were prepared at a concentration of 1 g/l (w/v) in 18 mΩ water in a sterile acid washed 50 ml volumetric flask. SRHA stock solution [1 g/l] was sonicated at 38 kHz ±10 % in a bath sonicator (Pulsatron 325, Kerry) for two 8 minute bursts, with ~10 seconds manual shaking in between. Stocks were prepared and used on the day of the test.
The impact of SRHA on the toxicity of silver on the toxicity of silver (ionic and nanoparticle) on algae was assessed using 6 test conditions for each media (made in 160 ml, or 320 ml for unmodified toxicant free controls, and separated into three ~50 ml replicates as in Section 3.4.3.) as follows. Unmodified toxicant free medium control (6 replicates) comprised of 80 ml toxicant free 18 mΩ water, 80 ml double strength medium (OECD medium/JM) and 160 ml exponentially growing R. subcapitata diluted to 1 x 10^5 cells/ml. 5 mg/l SRHA toxicant free medium control (3 replicates) comprised of 0.8 ml SRHA stock [1 g/l] , 39.2 ml toxicant free 18 mΩ water, 40 ml double strength medium (OECD medium/JM) and 80 ml exponentially growing R. subcapitata diluted to 1 x 10^5 cells/ml. Silver at 80% effective concentration (EC80) (3 replicates) comprised of 40 ml of the test substance (NM300K, AgNO3) at four times the desired test concentration in 18 mΩ water, 40 ml double strength medium (OECD medium/JM), and 80 ml exponentially growing R. subcapitata diluted to 1 x 10^5 cells/ml. Silver at 80% effective concentration (EC80) in medium with 5 mg/l SRHA (3 replicates) comprised of 0.8 ml SRHA stock [1 g/l], 19.2 ml toxicant free 18 mΩ water, 20 ml of the test substance (NM300K, AgNO3) at eight times the desired test concentration in 18 mΩ water, 40 ml double strength medium (OECD medium/JM), and 80 ml exponentially growing R. subcapitata diluted to 1 x 10^5 cells/ml. Silver at 80% effective concentration (EC80) in medium with 50 mg/l SRHA (3 replicates) comprised of 8 ml SRHA stock [1 g/l], 12 ml toxicant free 18 mΩ water, 20 ml of the test substance (NM300K, AgNO3) at eight times the desired test concentration in 18 mΩ water, 40 ml double strength medium (OECD medium/JM), and 80 ml exponentially growing R. subcapitata diluted to 1 x 10^5 cells/ml. All test conditions were mixed in the order – base medium (with or without SRHA), followed by toxicant (or 18 mΩ water for controls), followed by algal culture.

pH was measured at 0 and 72 hours in each flask using a pH probe (Orion ROSS Ultra, Thermoscientific) connected to an electrochemistry meter (Orion Star Plus, Thermo Scientific). Test duration was 72 hours, following the same OECD protocol as described in Section 3.4.3.

3.4.5. Speciation modelling

Dissolved silver speciation modelling in both media (including humic and pH modifications) was performed using Visual Minteq v3.0 software (J.P. Gustafsson, Stockholm, 2011). This software allows chemical equilibrium calculations to be made by inserting all chemical medium components into the model and calculating the resulting re-partitioning of compounds. The software has been used in many studies where speciation is of interest,
including those of ionic silver and nanomaterials (Liu et al., 2011; Chen et al., 2013; Hedberg et al., 2014; Loza et al., 2014). For NM300K, calculations were made using the mean measured dissolved silver (all species) concentration at 0 and 72 hours as determined using ICP-MS in Section 2.5.1. For AgNO₃, calculations were made under the assumption that all silver was in dissolved form in the media. Chemical media components of JM and OECD medium used in speciation modelling can be found in Appendix 1. Humic acid additions were modelled on the assumption that all SRHA was dissolved, using the Stockholm Humic Model (Gustafsson, 2001).

3.4.6. Statistical analysis

Details of statistical testing software and procedures can be found in Section 2.4.5. *R. subcapitata* growth rate was compared at each timepoint in both media (i.e. all control treatments). Any control flasks showing no growth (i.e. no positive change in cell density over time) were removed from growth rate and dependent concentration response analyses (i.e. a total of three control replicates in one OECD medium/humic acid/AgNO₃ study, two control (toxicant and humic free) replicates in one JM/humic acid/NM300K study, and one pH 8 toxicant replicate in one JM/pH/AgNO₃ study). For concentration-response data, a probit model was fitted to growth inhibition data generated at each timepoint. EC₅₀ (μg/l) values were derived and compared between both media and timepoints, for NM300K and AgNO₃.

Due to poor chlorophyll-acetone extraction in the study of the influence of SRHA on AgNO₃ toxicity in JM, these results were derived from *in vivo* Chlₐ measurements, a method that shows good correlation with extracted Chlₐ (Section 4.4.1.4.; Figure 4.3). The full implications of the use of different methods to determine algal growth rate will be discussed in Chapter 4.

In humic acid/pH studies *R. subcapitata* growth rate between toxicant free humic acid concentrations (0, 5 and 50 mg/l SRHA) and pH levels (unadjusted, pH 6 and pH 8) were compared at each timepoint. The effect of humic acid/pH on growth inhibition were analysed at each timepoint, and compared to their appropriate toxicant free controls.

3.5. Results

For the purposes of comparison all stated silver concentrations, regardless of type (i.e. NM300K or AgNO₃), are expressed in mass of silver per litre. Dispersant only (NM300K-DIS) toxicity studies showed no toxicity at the highest equivalent concentration of NM300K studied in both media (i.e. EC₅₀ > 150 μg/l in OECD, and > 60 μg/l in JM). As a result, NM300K-DIS was considered to be non-toxic to *R. subcapitata*, and was not further studied in humic acid/pH experiments.
3.5.1. Toxicity of silver nanoparticles and ions in OECD medium

The average *R. subcapitata* control growth rate in OECD medium was 1.80 (±0.03), 1.81 (±0.02), 1.51 (±0.02) d⁻¹ at 24, 48 and 72 h, respectively (Figure 3.1).

![Figure 3.1](image)

**Figure 3.1:** Experimental growth rate of *R. subcapitata* in control cultures of OECD medium (black) and JM (white). Data expressed as mean toxicant free control growth and standard error of the mean (nOECD medium = 92; nJM = 63). Letters denote significant differences within each timepoint (Mann-Whitney U; p < 0.05); bars not sharing the same letter were significantly different.

In OECD medium both NM300K and AgNO₃ induced a concentration dependent decrease in algal growth at all time points investigated (Figure 3.2, Table 3.1 and Figure 3.3, Table 3.2 for NM300K and AgNO₃ respectively). The greatest decrease in algal growth was observed at 24 hours for both forms of silver, with a reduction in toxicity (higher EC₅₀) observed with increasing test duration. The EC₅₀ (50 % inhibition of algal growth in relation to toxicant free control cultures) values for NM300K were 50.6 (±1.17), 80.33 (±2.40) and 130.65 (±1.73) µg/l at 24, 48 and 72 hours, respectively (Figure 3.2, Table 3.1). AgNO₃ was between 27-36 times (depending on timepoint) more toxic (lower EC₅₀) than NM300K, with EC₅₀ values of 1.84 (±3.11), 2.22 (±0.06) and 4.86 (±0.11) µg/l at 24, 48 and 72 hours, respectively (Figure 3.3, Table 3.2). When NM300K toxicity was expressed in terms of mean dissolved silver (assessed using ICP-MS) (Chapter 2, Table 2.2; Section 3.5.4.), EC₅₀ concentrations were not significantly different to that of AgNO₃; 1.65 (±0.04), 2.62 (±0.08) and 4.27 (±0.06) µg/l for 24, 48 and 72 hours, respectively.
Figure 3.2: Impact of NM300K [30-150 µg/l Ag] on *R. subcapitata* growth rate inhibition in OECD medium, at 24 hours (black circles) 48 hours (grey squares and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 3).

Table 3.1: Probit modelled effective concentrations of NM300K [30-150 µg/l Ag] on *R. subcapitata* growth rate (24, 48, and 72 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model of total Ag induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 3). Superscript letters denote significant differences in EC50 between each timepoint (Kruskal-Wallis; p < 0.05); EC50 values not sharing the same superscript letter were significantly different.
Figure 3.3: Impact of AgNO₃ [0.2-20 µg/l] on *R. subcapitata* growth rate inhibition in OECD medium, at 24 hours (black circles) 48 hours (grey squares and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Table 3.2: Probit modelled effective concentrations of AgNO₃ [0.2-20 µg/l] on *R. subcapitata* growth rate (24, 48, and 72 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model of total Ag induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 1, one experiment with three replicates). A Kruskal-Wallis test between each timepoint found significant differences (p = 0.027) in EC₅₀, but a Mann-Whitney U test was unable to identify specific differences.

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<th>48 hour</th>
<th>72 hour</th>
</tr>
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<td>1.40 (±0.07)</td>
<td>2.68 (±0.14)</td>
</tr>
<tr>
<td>20</td>
<td>1.66 (±6.71)</td>
<td>1.68 (±0.05)</td>
<td>3.43 (±0.12)</td>
</tr>
<tr>
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<td>2.22 (±0.06)</td>
<td>4.86 (±0.11)</td>
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<td>80</td>
<td>2.02 (±0.49)</td>
<td>2.75 (±0.11)</td>
<td>6.29 (±0.14)</td>
</tr>
</tbody>
</table>
3.5.1.1. Toxicity of silver nanoparticles and ions in OECD medium with humic acid

In OECD control treatments without humic acid or toxicant, *R. subcapitata* growth rate was 1.60 (±0.05), 1.74 (±0.04) and 1.45 (±0.04) d⁻¹ for 24, 48 and 72 hours respectively (Figure 3.4). Compared to humic acid and toxicant free treatments, humic acid concentration resulted in a significantly lower growth rate at 24 and 48 hours (F = 16.65, p < 0.001 and F = 28.05, p < 0.001). At these timepoints, treatments containing humic acid (5 or 50 mg/l SRHA) were not significantly different from each other, with a 24 hour growth rate of 1.28 (±0.05) and 1.25 (±0.03) d⁻¹ and 48 hour growth rate of 1.44 (±0.05) and 1.32 (±0.02) d⁻¹, for 5 and 50 mg/l SRHA respectively. At 72 hours however, only 50 mg/l SRHA resulted in a significantly lower growth rate (F = 8.55, p = 0.001), with a growth rate of 1.18 (±0.04) compared to 1.38 (±0.05) in OECD medium containing 5 mg/l SRHA. Regardless of differences in growth rate, treatments containing toxicant (either 190 µg/l NM300K or 6 µg/l AgNO₃) were always compared to their corresponding humic acid control (0, 5 or 50 mg/l SRHA), and expressed as percent inhibition (Figures 3.5 and 3.6).

![Graph showing growth rate comparison](image)

**Figure 3.4:** Experimental growth rate of *R. subcapitata* in control cultures of OECD medium (black, humic acid free), and medium containing 5 mg/l (grey) and 50 mg/l (white) Suwanee River humic acid. Data expressed as mean toxicant free control growth and standard error of the mean (n_control = 24; n_SRHA = 12). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.
For OECD medium containing 190 µg/l NM300K (Figure 3.5) at 24 hours, a Kruskal-Wallis test, followed by multiple Mann-Whitney U post hoc testing, revealed a significant difference in growth inhibition between all SRHA treatments ($H = 23.14, p < 0.001$) with a growth inhibition of 202.03 ($±6.62$), 156.66 ($±4.04$) and 31.98 ($±0.95$) % for 0, 5 and 50 mg/l SRHA respectively. Results therefore indicate that the presence of SRHA decreased the toxicity of NM300K. The same concentration dependent effect was observed for humic acid at 48 hours ($H = 21.08, p < 0.001$), with a growth inhibition of 190 µg/l NM300K in OECD medium of 145.59 ($±6.67$) 117.02 ($±5.04$) and 46.00 ($±4.59$) % for 0, 5 and 50 mg/l SRHA respectively. By 72 hours, only 50 mg/l SRHA had a significant effect on reducing NM300K toxicity to *R. subcapitata* in OECD medium ($F = 25.53, p < 0.001$), relative to humic acid free or 5 mg/l SRHA, with a growth inhibition of 111.63 ($±5.56$), 96.07 ($±5.48$) and 48.12 ($±8.24$) % at 0, 5 and 50 mg/l SRHA respectively. As observed in concentration-response studies, NM300K induced growth inhibition decreased significantly over time for both humic acid free ($F = 52.48, p < 0.001$) and OECD medium containing 5 mg/l SRHA ($F = 39.58, p < 0.001$). However in OECD medium containing 50 mg/l SRHA, no significant difference in NM300K growth inhibition was observed over time (24, 48, and 72 hours).
Figure 3.5: Impact of NM300K [190 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on R. subcapitata growth inhibition, in OECD medium. NM300K growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different.

For OECD medium containing 6 µg/l AgNO₃ (Figure 3.6) at 24 hours, a Kruskal-Wallis test, followed by multiple Mann-Whitney U post hoc testing, revealed a significant difference in growth inhibition between SRHA treatments (H = 6.49, p = 0.039) with a growth inhibition of 138.89 (±6.60), 119.80 (±10.50) and 40.67 (±0.95) % for 0, 5 and 50 mg/l SRHA respectively. This suggests that SRHA reduced the toxicity of AgNO₃. However when a Mann-Whitney U test was applied to identify pairwise significant differences, none were found. This result is likely due to the fact that the sample size was small (one experiment with three replicates) and the test used was not sufficiently powerful enough to detect any differences. In the same test, at 48 hours, growth inhibition of 6 µg/l AgNO₃ in OECD medium containing 5 mg/l SRHA (91.66 ±2.07 %) was not significantly different to humic acid free medium (81.45 ±5.76 %).

However 50 mg/l SRHA significantly reduced AgNO₃ toxicity to R. subcapitata at this timepoint (F = 50.36, p < 0.001), with a growth inhibition of 41.78 (±1.90) %. By 72 hours, the presence of humic acid had no significant effect on 6 µg/l AgNO₃ toxicity to R. subcapitata in OECD medium, relative to humic acid free medium, with a growth inhibition of 39.76 (±7.45), 55.36 (±3.85) and 25.34 (±2.38) % at 0, 5 and 50 mg/l SRHA respectively. However 5 mg/l SRHA
resulted in a significantly higher growth inhibition relative to 50 mg/l SRHA (F = 8.63, p = 0.017). As observed in both concentration response studies and humic acid studies with NM300K, humic acid free OECD medium containing 6 µg/l AgNO₃ showed a significant time dependent reduction in toxicity at all timepoints (F = 56.19, p < 0.001). However both 5 and 50 mg/l SRHA only showed a significant reduction in AgNO₃ toxicity at 72 hours (F = 24.40, p = 0.001 and F = 20.27, p = 0.002) respectively, relative to 24 and 48 hours.

![Graph](image)

**Figure 3.6:** Impact of AgNO₃ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* growth inhibition, in OECD medium. AgNO₃ growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different. A Kruskal-Wallis test at 24 hours found significant differences (p < 0.001) in growth inhibition between humic acid conditions, but a Mann-Whitney U test was unable to identify specific differences.

In OECD medium, it is clear that the toxicity reducing effect of SRHA is more pronounced at higher concentrations of humic acid. For 190 µg/l NM300K at 72 hours, growth inhibition was reduced from 111.63 (±5.56) % in humic acid free medium to 48.12 (±8.24) % in medium containing 50 mg/l SRHA, representing over a two-fold reduction in toxicity (Figure 3.5). However in AgNO₃ studies, this effect was less pronounced, with no significant reduction in toxicity by the end of the test (Figure 3.6). This effect may be due to the fact that in humic
acid free OECD medium, 6 µg/l AgNO₃ only resulted in a 72 hour growth inhibition of 39.76 (±7.45) %, which may have reduced the ability of the test to detect differences between humic acid levels. Despite this, broadly similar patterns of toxicity were observed between NM300K and AgNO₃ in OECD medium containing SRHA.

3.5.1.2. Toxicity of silver nanoparticles and ions in OECD medium at pH 6 and 8

Unbuffered OECD medium showed little change in pH from 7.5 (< 0.1 units) over the 72 hour test duration, and 3.5 mM MOPS buffer retained the pH value within 0.65 and 0.19 units of the starting (i.e. 0 hour) pH for pH 6 and 8, respectively (Figure 3.7). There was a significant difference in conductivity between both MOPS buffered and unbuffered OECD medium (toxicant free controls) (F = 371.6, p < 0.001) (Figure 3.8). At 72 hours, conductivity was 126.42 (±0.46), 146.4 (±0.29) and 251.73 (±0.72) µS/m in unbuffered, pH 6 and pH 8 OECD medium, respectively. Conductivity decreased in all media over the test duration (Figure 3.8).

![Figure 3.7: Daily pH measurements of control cultures (toxicant free) of R. subcapitata in unbuffered OECD medium (black circles, solid line), and OECD medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey squares, dotted line) and pH 8 (white squares, dashed line). Data expressed as mean toxicant free control pH and standard error of the mean (error bars smaller than markers; n = 3).](image-url)
Figure 3.8: Daily conductivity measurements of control cultures (toxicant free) of *R. subcapitata* in unbuffered OECD medium (black circles), and OECD medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey squares) and pH 8 (white squares). Data expressed as mean toxicant free control pH and standard error of the mean (error bars smaller than markers; n = 3).

In OECD medium control treatments without pH modification or toxicant, *R. subcapitata* growth rate was 1.70 (±0.05), 1.88 (±0.03) and 1.55 (±0.02) d⁻¹ for 24, 48 and 72 hours respectively (Figure 3.9). Compared to unmodified/toxicant free treatments, pH 6 resulted in a significantly higher growth rate at 24 (F = 9.21, p < 0.001) and 48 (F = 13.04, p < 0.001) hours, with a growth rate of 1.93 (±0.04) and 2.03 (±0.03) d⁻¹. However by 72 hours, *R. subcapitata* growth rate in pH 6 OECD medium was 1.58 (±0.03) d⁻¹, and was not significantly different to either unmodified or pH 8 medium. Growth rate in toxicant free, pH 8 OECD medium was not significantly different from unmodified medium at any timepoint, with a growth rate of 1.60 (±0.05), 1.78 (±0.04) and 1.52 (±0.02) d⁻¹ at 24, 48 and 72 hours respectively. As in humic acid studies, treatments containing toxicant (either 190 µg/l Ag NM300K or 6 µg/l Ag AgNO₃) were always compared to their corresponding pH control (unmodified, pH 6 and pH 8), and expressed as percent inhibition (Figures 3.10 and 3.11).
Figure 3.9: Experimental growth rate of *R. subcapitata* in control cultures of OECD medium (black, unbuffered), and medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey) and pH 8 (white). Data expressed as mean toxicant free control growth and standard error of the mean (ncontrol = 21; nSRHA = 12). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

For OECD medium containing 190 µg/l NM300K (Figure 3.10) at 24 hours, medium buffered to pH 8 caused a significant increase in growth inhibition (F = 15.76, p < 0.001), compared to both unbuffered and pH 6 medium; with a growth inhibition of 147.96 (±13.42), 136.11 (±22.70) and 267.80 (±17.73) % for unbuffered, pH 6 and pH 8 medium respectively. A similar significant difference was observed between pH conditions at 48 (F = 3.69, p = 0.04) and 72 (F = 4.14, p = 0.029) hours; that is, pH 8 medium caused a significantly higher growth inhibition of NM300K to *R. subcapitata*, relative to unbuffered medium. Comparatively at 48 or 72 hours, pH 6 OECD medium was not significantly different to unbuffered or pH 8 medium. At 48 hours, NM300K growth inhibition was 78.38 (±12.13), 117.45 (±23.87) and 165.36 (±13.61) % for unbuffered, pH 6 and pH 8 medium respectively. At 72 hours, growth inhibition was 59.80 (±11.17), 84.69 (±21.53) and 123.41 (±13.15) % for unbuffered, pH 6 and pH 8 medium respectively. As in concentration-response and humic studies, NM300K induced growth inhibition decreased significantly over time for unbuffered (F = 12.09, p < 0.001) and pH 8 (F = 24.62, p < 0.001) OECD medium. However in OECD medium buffered to pH 6, no significant difference in NM300K growth inhibition was observed over time. Overall, these results indicate that the toxicity of NM300K is higher at pH 8, especially over shorter exposures.
Figure 3.10: Impact of pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on NM300K [190 µg/l Ag] toxicity to *R. subcapitata*, in OECD medium. NM300K growth inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

For OECD medium containing 6 µg/l AgNO₃ (Figure 3.11) at 24 hours, medium buffered to pH 8 caused a significant increase in growth inhibition (F = 16.48, p = 0.004), compared to both unbuffered and pH 6 medium; with a growth inhibition of 116.61 (±6.65), 100.27 (±1.78) and 258.25 (±53.87) % for unbuffered, pH 6 and pH 8 medium respectively. At 48 hours, all three test conditions (unbuffered, pH 6 and pH 8) resulted in significantly different AgNO₃ induced toxicity to *R. subcapitata* (F = 77.21, p < 0.001), with a growth inhibition of 72.45 (±3.31), 87.10 (±1.77) and 139.89 (±6.20) % for unbuffered, pH 6 and pH 8 medium respectively. At 72 hours, only pH 8 OECD medium resulted in a significantly higher growth inhibition (F = 42.97, p < 0.001), relative to unbuffered and pH 6 medium; with a growth inhibition of 34.59 (±4.97), 49.56 (±2.95) and 94.10 (±5.79) % for unbuffered, pH 6 and pH 8 medium respectively. As in concentration-response, humic studies, and pH studies with NM300K, AgNO₃ induced growth inhibition decreased significantly over time for unbuffered (F = 63.23, p < 0.001) and pH 8 (F = 7.24, p = 0.025) OECD medium. However, unlike results for NM300K in OECD medium, pH 6 medium also showed a significant time-dependent reduction
in AgNO₃ growth inhibition (F = 138.54, p < 0.001). Similar to results obtained for NM300K, results indicate that toxicity of AgNO₃ is enhanced at pH 8, especially in shorter exposures.

![Figure 3.1](image)

**Figure 3.11**: Impact of pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on AgNO₃ [6 µg/l Ag] toxicity to *R. subcapitata*, in OECD medium. NM300K growth inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

In OECD medium, it is clear that the toxicity of silver (both forms) is enhanced at pH 8. For 190 µg/l NM300K at 72 hours, growth inhibition was enhanced from 59.80 (±11.17) % in unbuffered medium to 123.41 (±13.15) % in medium buffered to pH 8, representing a two fold increase in toxicity (Figure 3.10). A similar effect was observed with AgNO₃, with a 72 hour growth inhibition 34.59 (±4.97) % in unbuffered medium, compared to 94.10 (±5.79) % at pH 8 (Figure 3.11); representing almost a threefold increase in toxicity. At pH 6, though a slight increase in toxicity was observed in both NM300K (Figure 3.10) and AgNO₃ (Figure 3.11), this effect was mostly not significantly higher than unbuffered OECD medium (the only exception being for 6 µg/l AgNO₃ at 48 hours).
3.5.2. Toxicity of silver nanoparticles and ions in JM

*R. subcapitata* control growth rate in JM was 1.75 (±0.05), 1.84 (±0.03), 1.72 (±0.03) d\(^{-1}\) at 24, 48 and 72 hours respectively (Figure 3.1). At the three timepoints measured, growth rate in JM was only significantly higher than in OECD medium at 72 hours (*W* = 6886.0, p < 0.001), attributable to the higher nutrient content of this medium which may increase the onset of growth rate reduction as a result of nutrient limitation.

As in OECD medium, both NM300K and AgNO\(_3\) induced a concentration dependent decrease in algal growth in JM at all time points investigated (Figure 3.12, Table 3.3 and Figure 3.13, Table 3.4 for NM300K and AgNO\(_3\) respectively). A similar time dependent reduction in silver (ionic and nanoparticle) toxicity (increasing EC\(_{50}\)) was also observed (Figure 3.12, Table 3.3 and Figure 3.13, Table 3.4 for NM300K and AgNO\(_3\) respectively); with the greatest impact on algal growth observed at 24 hours. The EC\(_{50}\) values for NM300K were 33.34 (±0.35), 42.75 (±0.39) and 54.18 (±0.55) µg/l at 24, 48 and 72 hours respectively (Figure 3.12, Table 3.3). When compared to total silver in nanoparticles, AgNO\(_3\) was between 8-9 times (depending on timepoint) more toxic (lower EC\(_{50}\)) than NM300K, with EC\(_{50}\) values of 3.61 (±0.24), 5.33 (±0.14) and 6.93 (±0.16) µg/l at 24, 48 and 72 hours respectively (Figure 3.13, Table 3.4). When NM300K toxicity was expressed in terms of mean ICP-MS dissolved silver (Table 3.5 and Chapter 2, Table 2.2), EC\(_{50}\) concentrations were lower than that of AgNO\(_3\); 1.59 (±0.02), 2.04 (±0.02) and 2.59 (±0.03) µg/l for 24, 48 and 72 hours, respectively. Unlike OECD medium, the difference in EC\(_{50}\) as a result of mean dissolved silver from NM300K and AgNO\(_3\) was significant (p < 0.001), indicating that NM300K and AgNO\(_3\) behave differently within JM.
Figure 3.12: Impact of NM300K [20-60 µg/l Ag] on *R. subcapitata* growth rate inhibition in JM, at 24 hours (black circles) 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 3).

Table 3.3: Probit modelled effective concentrations of NM300K [20-60 µg/l Ag] on *R. subcapitata* growth rate (24, 48, and 72 hour) in JM. Data expressed as mean effective concentration derived from a probit model of total Ag induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 3). Superscript letters denote significant differences in EC$_{50}$ between each timepoint (One way ANOVA; p < 0.05); EC$_{50}$ values not sharing the same superscript letter were significantly different.

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<th>NM300K (µg/l Ag)</th>
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<th>72 hour</th>
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</table>
Figure 3.13: Impact of AgNO$_3$ [1-30 µg/l Ag] on *R. subcapitata* growth rate inhibition in JM, at 24 hours (black circles) 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Table 3.4: Probit modelled effective concentrations of AgNO$_3$ [1-30 µg/l Ag] on *R. subcapitata* growth rate (24, 48, and 72 hour) in JM. Data expressed as mean effective concentration derived from a probit model of total Ag induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 1, one experiment with three replicates). A Kruskal-Wallis test between each timepoint found significant differences (p = 0.039) in EC$_{50}$, but a Mann-Whitney U test was unable to identify specific differences.
3.5.2.1. Toxicity of silver nanoparticles and ions in JM with humic acid

*R. subcapitata* growth rate was 1.89 (±0.10), 1.88 (±0.05) and 1.80 (±0.04) d⁻¹ for 24, 48 and 72 hours respectively in JM controls in the absence of SRHA or silver (Figure 3.14). In control treatments containing 5 mg/l SRHA growth rate was 1.93 (±0.12), 1.89 (±0.07), 1.77 (±0.08) d⁻¹ for 24, 48 and 72 hours respectively; none of which were significantly different to humic acid free controls. Unlike in OECD medium experiments, humic acid concentration had less of an effect on lowering growth rate of NM300K exposed algae than in JM. The only significant effect was observed at 48 hours for 50 mg/l SRHA (F = 3.62, p = 0.035), with a significantly lower growth rate of 1.67 (±0.09) d⁻¹. *R. subcapitata* growth rate was 1.56 (±0.12) and 1.60 (±0.09) d⁻¹ in toxicant free JM containing 50 mg/l SRHA, at 24 and 72 hours respectively. As with OECD medium, treatments containing toxicant (either 80 µg/l Ag NM300K or 11 µg/l Ag AgNO₃) were always compared to their corresponding humic acid control (0, 5 or 50 mg/l SRHA), and expressed as percent inhibition (Figures 3.15 and 3.16).

![Figure 3.14](image)

*Figure 3.14*: Experimental growth rate of *R. subcapitata* in control cultures of JM (black, humic acid free), and medium containing 5 mg/l (grey) and 50 mg/l (white) Suwanee River humic acid (SRHA). Data expressed as mean toxicant free control growth and standard error of the mean (n<sub>control</sub> = 22; n<sub>SRHA</sub> = 12). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

For JM containing 80 µg/l NM300K (Figure 3.15) at 24 hours, only algae cultured with 50 mg/l SRHA resulted in a significant reduction in growth inhibition (F = 45.03, p < 0.001)
relative to humic acid free and 5 mg/l SRHA JM medium, with a growth inhibition of 133.73 (±6.58), 104.35 (±7.99) and 24.00 (±10.40) % for 0, 5 and 50 mg/l SRHA respectively. The same significant difference was observed between humic acid conditions at 48 hours (F = 29.46, p < 0.001), with a growth inhibition of 80 µg/l NM300K in JM of 108.93 (±8.86), 76.44 (±8.27) and 18.67 (±8.12) % for 0, 5 and 50 mg/l SRHA respectively. By 72 hours, both 5 and 50 mg/l SRHA had a significant effect on reducing NM300K toxicity to *R. subcapitata* in JM (F = 46.27, p < 0.001) relative to humic acid free medium, with a growth inhibition of 82.37 (±5.34), 55.55 (±5.81) and 9.60 (±5.04) % at 0, 5 and 50 mg/l SRHA respectively. As in concentration-response studies and humic studies with OECD medium, NM300K induced growth inhibition decreased significantly over time for both humic acid free (F = 13.17, p < 0.001) and 5 mg/l SRHA (F2,24 = 10.83, p < 0.001) JM. Similarly to OECD medium (Figure 3.4), timepoint had no significant effect on algal growth inhibition in JM containing NM300K and 50 mg/l SRHA. The results therefore suggest that SRHA reduced the toxicity of NM300K to algae in JM.

**Figure 3.15**: Impact of NM300K [80 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* growth inhibition, in JM. NM300K growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.
For JM containing 11 µg/l AgNO₃ (Figure 3.16), a Kruskal-Wallis test, followed by multiple Mann-Whitney U post hoc testing, revealed a significant difference in growth inhibition between SRHA treatments at 24 (H = 6.49, p = 0.039), 48 (H = 7.20, p = 0.027) and 72 (H = 6.49, p = 0.039) hours. At 24 hours in JM a growth inhibition of 180.64 (±0.38), 173.01 (±4.50) and 81.84 (±2.75) % was observed for 0, 5 and 50 mg/l SRHA respectively. At 48 hours a growth inhibition of 177.90 (±1.80), 163.22 (±0.71) and 61.87 (±2.99) % was observed for 0, 5 and 50 mg/l SRHA respectively. Finally at 72 hours, a growth inhibition of 154.78 (±2.03), 150.79 (±1.14) and 54.84 (±4.09) was observed for 0, 5 and 50 mg/l SRHA respectively. Despite finding significant differences between the humic acid concentrations, post hoc testing using Mann-Whitney U tests were unable to identify pairwise significant differences. As with OECD AgNO₃ humic studies at 24 hours, this result is likely due to the fact that the sample size was small (one experiment with three replicates) and the test used was not sufficiently powerful enough to detect any differences. As observed in both concentration response studies and humic acid studies with NM300K, humic acid free JM containing 11 µg/l AgNO₃ showed a significant time dependent reduction in toxicity, but only at 72 hours (F = 80.92, p < 0.001). A similar pattern of toxicity reduction was also observed in JM containing 11 µg/l AgNO₃ and 5 mg/l SRHA (i.e. only a significant reduction in growth inhibition at 72 hours; F = 17.20, p = 0.003). However in JM containing 11 µg/l AgNO₃ and 50 mg/l SRHA, growth inhibition was significantly reduced after 48 hours, but showed no further reduction in toxicity by 72 hours (F = 17.73, p = 0.003).
Figure 3.16: Impact of AgNO₃ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on R. subcapitata growth inhibition, in OECD medium. AgNO₃ growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). A Kruskal-Wallis test at each timepoint found significant differences (p < 0.001) in growth inhibition between humic acid conditions, but a Mann-Whitney U test was unable to identify specific differences.

Similar to OECD medium, it is evident that the toxicity reducing effect of SRHA in JM is more pronounced at higher concentrations of humic acid. For 80 µg/l NM300K at 72 hours, growth inhibition was reduced from 82.37 (±5.34) % in humic acid free medium to 9.60 (±5.04) % in medium containing 50 mg/l SRHA, representing over an eight-fold reduction in toxicity (Figure 3.15). However in AgNO₃ studies however, this effect was less pronounced; 154.78 (±2.03) % in humic acid free medium to 54.84 (±4.09) % in medium containing 50 mg/l SRHA, representing nearly a three-fold reduction (Figure 3.16). Despite this, broadly similar patterns of toxicity were observed between NM300K and AgNO₃ in JM containing SRHA.

3.5.2.2. Toxicity of silver nanoparticles and ions in JM at pH 6 and 8

Unbuffered JM showed a change in pH of around 2 units (6.9 - 8.9) over the 72 hour test duration, however the application of 3.5 mM MOPS buffer retained the pH within 0.76 and 0.73 units of the starting (i.e. 0 hour) pH for JM medium buffered to pH 6 and 8
respectively (Figure 3.17). By 72 hours, the pH in unbuffered JM control cultures containing *R. subcapitata* had risen to a level that was not significantly different from the pH 8 test condition; despite all three control test conditions being significantly different at 0 hours ($F = 5755.44$, $p < 0.001$) (Figure 3.17). There was a significant difference in conductivity between unbuffered JM medium and medium buffered to pH 6 and 8 (toxicant free); 72 hour conductivity was 219.94 (±1.37), 214.36 (±3.6) and 350.89 (±2.64) μS/m in unbuffered, pH 6 and pH 8 JM respectively. As in OECD medium, conductivity decreased in all media over the test duration (Figure 3.18). In addition, pH 8 JM had a significantly higher conductivity than either unbuffered or pH 6 ($F = 1366.87$, $p < 0.001$).

**Figure 3.17:** Daily pH measurements of control cultures (toxicant free) of *R. subcapitata* in unbuffered JM (black circles, solid line), and JM buffered (using with 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey squares, dotted line) and pH 8 (white squares, dashed line). Data expressed as mean toxicant free control pH and standard error of the mean (error bars smaller than markers; $n = 3$).
Figure 3.18: Daily conductivity measurements of control cultures (toxicant free) of *R. subcapitata* in unbuffered OECD medium (black circles), and OECD medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey squares) and pH 8 (white squares). Data expressed as mean toxicant free control pH and standard error of the mean (error bars smaller than markers; n = 3).

In JM control treatments without pH modification or toxicant, *R. subcapitata* growth rate was 1.90 (±0.03), 1.96 (±0.02) and 1.83 (±0.02) d⁻¹ at 24, 48 and 72 hours respectively (Figure 3.19). Compared to unmodified/toxicant free treatments, pH 8 resulted in a significantly lower growth rate only at 72 hours (F = 3.55, p < 0.037), with a growth rate of 1.76 (±0.03) d⁻¹. At 24 and 48 hours at pH 8 JM, *R. subcapitata* growth rate was 1.78 (±0.05) and 1.86 (±0.04) d⁻¹ respectively, and was not significantly different to either unmodified or pH 6 medium. Growth rate in toxicant free, pH 6 JM was not significantly different from unmodified medium at any timepoint, with a growth rate of 1.90 (±0.03), 1.97 (±0.02) and 1.82 (±0.01) d⁻¹ at 24, 48 and 72 hours respectively. As in other humic and pH studies presented here, treatments containing toxicant (either 80 µg/l Ag NM300K or 11 µg/l Ag AgNO₃) were always compared to their corresponding pH control (unmodified, pH 6 and pH 8), and expressed as percent inhibition (Figure 3.20 and 3.21).
Figure 3.19: Experimental growth rate of *R. subcapitata* in control cultures of JM (black, unbuffered), and medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey) and pH 8 (white). Data expressed as mean toxicant free control growth and standard error of the mean (*n*control = 21; *n*SRHA = 12). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

For JM containing 80 µg/l NM300K (Figure 3.20) at 24 hours, at pH 8 a significant increase in growth inhibition (F = 25.37, p < 0.001), compared to both unbuffered and pH 6 medium was observed; with a growth inhibition of 104.99 (±15.14), 58.84 (±14.71) and 199.87 (±14.27) % for unbuffered, pH 6 and pH 8 medium respectively. A similar significant difference in toxicity was observed between pH conditions at 48 hours (F = 13.25, p < 0.001) with a growth inhibition of 59.08 (±11.22), 28.95 (±9.92) and 98.88 (±10.30) % for unbuffered, pH 6 and pH 8 medium respectively. However at 72 hours, pH 8 JM containing 80 µg/l NM300K no longer showed a significant difference in toxicity to *R. subcapitata* in unbuffered JM. However, pH 6 JM containing NM300K resulted in a significantly lower growth inhibition at 72 hours; with a growth inhibition of 38.22 (±8.81), 14.90 (±5.07) and 69.78 (±7.75) % for unbuffered, pH 6 and pH 8 medium respectively. The data indicate that at a pH of 6, the toxicity of NM300K is reduced, whilst at pH 8 toxicity is enhanced. As in JM concentration-response and humic studies, NM300K induced growth inhibition decreased significantly over time for all three media conditions; unbuffered (F = 8.09, p = 0.002), pH 6 (F = 7.68, p = 0.003) and pH 8 (F = 30.66, p < 0.001).
Figure 3.20: Impact of NM300K [80 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-
morpholino)propanesulfonic acid) on *R. subcapitata* growth inhibition, in JM. NM300K growth inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

For JM containing 11 µg/l AgNO$_3$ (Figure 3.21) at 24 hours, medium buffered to pH 8 caused a significant increase in growth inhibition (F = 13.85, p = 0.009), compared to both unbuffered and pH 6 medium; with a growth inhibition of 188.57 (±8.20), 204.40 (±9.96) and 255.96 (±0.79) % for unbuffered, pH 6 and pH 8 medium respectively. However at both 48 and 72 hours, there was no significant difference in AgNO$_3$ induced growth inhibition to *R. subcapitata* between any of the pH conditions studied. At 48 hours growth inhibition was 161.36 (±4.38), 168.36 (±1.14) and 171.73 (±1.89) % for unbuffered, pH 6 and pH 8 medium respectively. At 72 hours growth inhibition was 124.14 (±4.50), 116.21 (±2.32) and 117.13 (±2.62) % for unbuffered, pH 6 and pH 8 medium respectively. As in both JM and OECD medium concentration-response and humic studies, AgNO$_3$ induced growth inhibition decreased significantly over time for all three media conditions; unbuffered (F = 29.40, p = 0.001), pH 6 (F = 55.67, p < 0.001) and pH 8 (F = 1308.78, p < 0.001).
Figure 3.21: Impact of AgNO₃ [11 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* growth inhibition, in JM. NM300K growth inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Unlike OECD medium, the effect of pH modification on silver toxicity in JM was less pronounced. For example at 72 hours in JM containing 80 µg/l NM300K, growth inhibition was significantly reduced from 38.22 (±8.81) % in unbuffered medium, to 14.90 (±5.07) % in medium buffered to pH 6, representing over a two-fold decrease in toxicity (Figure 3.20). Although JM buffered to pH 8 appeared to increase the toxicity of NM300K, this increase was only significantly greater than that observed in unbuffered medium at earlier timepoints (i.e. 24 and 48 hours, but not at 72 hours). With AgNO₃ however, there was no significant difference in test conditions as a result of pH except at 24 hours (Figure 3.21).

3.5.3. The effect of ICP-MS derived dissolved silver and resultant modelled speciation

In order to gain a better understanding of the possible contribution of dissolved silver speciation in both media to toxicity, concentration-response toxicity data were recalculated for NM300K in terms of ICP-MS derived mean total dissolved silver (Table 3.5; dissolution data presented in Chapter 2; Table 2.2). Once a mean dissolved silver concentration had been obtained, chemical speciation modelling was performed in each media to obtain partitioning
of total dissolved silver (Table 3.6 and 3.7). Finally, concentration response data were further recalculated on the basis of modelled free silver (Ag⁺) speciation in both media, for both NM300K (Table 3.8) and AgNO₃ (Table 3.9). For AgNO₃, which was not characterised using ICP-MS, total dissolved silver was assumed to be equal to total silver concentration (as AgNO₃ was considered as fully soluble in both media).

On the basis of NM300K mean ICP-MS derived dissolved silver in OECD medium (3.27 %) and JM (4.77 %), OECD medium showed a significantly higher EC₅₀ relative to JM, but only at 72 hours (F = 9.48, p = 0.007) (Table 3.5); this may be attributable to lower mean NM300K dissolution in OECD medium. On the basis of dissolved silver concentration (for both NM300K and AgNO₃) in OECD medium, the EC₅₀ value was not significantly different between NM300K and AgNO₃ at any timepoint (Table 3.3 and 3.5). In JM however, the EC₅₀ value was significantly higher for AgNO₃ compared to NM300K at 24 (F = 31.13, p < 0.001), 48 (F = 67.21, p < 0.001) and 72 (W = 33, p = 0.016) hours (Table 3.4 and 3.5), indicating a different pattern of toxicity in this medium. When dissolved silver speciation was modelled in both media, a large difference in silver partitioning was observed (Table 3.6 and 3.7). In OECD medium two silver species were present in almost equal proportions; silver chloride (AgCl(aq)) at 53.17 % and free ionic silver (Ag⁺) at 42.62 % (Table 3.6). However in JM, the most abundant silver species was Ag⁺, at 97.11 % (Table 3.7). Once silver concentrations were recalculated on the basis of both ICP-MS-derived and modelled dissolved silver speciation, and concentration response data were expressed in terms of Ag⁺ concentration alone, both NM300K (Table 3.8) and AgNO₃ (Table 3.9) showed a significantly higher toxicity (lower EC₅₀) in OECD medium. For NM300K, Ag⁺ EC₅₀ in OECD medium was significantly lower than JM at 24 (W = 47, p < 0.001) and 72 (F = 7.76, p < 0.013) hours, but not at 48 hours. Whereas for AgNO₃, Ag⁺ EC₅₀ in OECD medium was significantly lower at 24 (F = 26.90, p = 0.007), 48 (F = 188.77, p < 0.001) and 72 (F =271.03, p < 0.001) hours.

As with ICP-MS dissolved silver, on the basis of Ag⁺ concentration in OECD medium, the EC₅₀ value was not significantly different between NM300K and AgNO₃ (F = 0; p = 0.989; Table 3.8 and 3.9). In JM however, there was a larger and significant difference in the EC₅₀ values between silver forms (p < 0.001; Table 3.8 and 3.9), indicating a different pattern of toxicity in this medium. Regardless of comparisons both within and between media, and with both forms of silver, the EC₅₀ values always showed a significant time dependent increase from 24 to 72 hours (p < 0.001), though this effect differed in magnitude between the variables studied (i.e. medium, silver form and speciation).
Table 3.5: Probit model of effective concentrations (µg/l) of Ag as mean (0 and 72 hour) ICP-MS derived dissolved Ag from NM300K on R. subcapitata growth rate (24, 48, and 72 hour) in OECD medium (3.27 % dissolved silver) and JM (4.77 % dissolved silver). Data expressed as mean effective concentration, derived from a probit model of ICP-MS derived dissolved Ag induced growth inhibition (compared to toxicant free controls). Standard error of the mean is shown in parentheses (n = 3). Superscript letters denote significant differences in EC<sub>50</sub> between media, at each timepoint (One way ANOVA; p < 0.05); EC<sub>50</sub> not sharing the same superscript letter were significantly different.

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<td>72 hour</td>
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<td>80</td>
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<td>6.07 (±0.11)</td>
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<td>72 hour</td>
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Table 3.6: Dissolved silver species partitioning in OECD medium performed using Visual Minteq v3.0 software (J.P. Gustafsson, Stockholm, 2011). Speciation modelling was performed using a fixed concentration of silver (10 µg/l) and concentrations of OECD medium salts provided in Appendix 1. Speciation expressed as a percentage of total silver (99.99 % of total silver accounted for due to rounding errors).

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<tr>
<td>AgCl&lt;sup&gt;-&lt;/sup&gt;</td>
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<tr>
<td>AgSO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0.04</td>
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<tr>
<td>AgNH&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
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Table 3.7: Dissolved silver species partitioning in JM performed using Visual Minteq v3.0 software (J.P. Gustafsson, Stockholm, 2011). Speciation modelling was performed using a fixed concentration of silver (10 µg/l) and concentrations of JM salts provided in Appendix 1. Speciation expressed as a percentage of total silver (100 % of total silver accounted for).

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<td>AgNO₃(aq)</td>
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Table 3.8: Probit model of effective concentrations (µg/l) of Ag as predicted Ag⁺ (using Visual Minteq v3.0 software; J.P. Gustafsson, Stockholm, 2011) combined with ICP-MS derived mean (0 and 72 hour) dissolved Ag, from NM300K (20-150 µg/l Ag in OECD medium; 20-60 µg/l Ag in JM) on R. subcapitata growth rate (24, 48, and 72 hour) in OECD medium (3.27 % dissolved silver) and JM (4.77 % dissolved silver). Data expressed as mean effective concentration, derived from a probit model of modelled Ag⁺ from ICP-MS derived dissolved Ag induced growth inhibition (compared to toxicant free controls). Standard error of the mean is shown in parentheses (n = 3). Superscript letters denote significant differences in EC₅₀ between media, at each timepoint (24 hours: Mann-Whitney U; 72 hours: One way ANOVA; p < 0.05); EC₅₀ not sharing the same superscript letter were significantly different.

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<td>Not achieved</td>
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Table 3.9: Probit model of effective concentrations (µg/l) of Ag as predicted Ag⁺ (using Visual Minteq v3.0 software; J.P. Gustafsson, Stockholm, 2011) from AgNO₃ (0.2-20 µg/l Ag in OECD medium; 1-30 µg/l Ag in JM; full dissolution assumed) on R. subcapitata growth rate (24, 48, and 72 hour) in OECD medium (42.62 % of total silver as Ag⁺) and JM (97.11 % of total silver as Ag⁺). Data expressed as mean effective concentration, derived from a probit model of modelled Ag⁺ induced growth inhibition (compared to toxicant free controls). Standard error of the mean is shown in parentheses (n = 1, one experiment with three replicates). Superscript letters denote significant differences in EC₅₀ between media, at each timepoint (One way ANOVA; p < 0.05); EC₅₀ not sharing the same superscript letter were significantly different.

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<td>3.51 (±0.24)⁴</td>
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<td>6.73 (±0.18)⁴</td>
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</table>

Dissolved silver speciation was also modelled in media containing SRHA (5 and 50 mg/l) and at different pH (6 and 8). In OECD medium, SRHA reduced Ag⁺ and AgClₐq in a concentration dependent manner, whilst increasing SRHA complexation; 1.02 % and 16.63 % of dissolved silver was predicted to have complexed with 5 and 50 mg/l SRHA, respectively (Table 3.10). In JM medium, containing less AgClₐq, SRHA reduced Ag⁺ in a concentration dependent manner. A higher proportion of dissolved silver was bound to SRHA in JM than in OECD medium, with 2.19 % and 21.16 % complexed with 5 and 50 mg/l SRHA, respectively.
Table 3.10: Dissolved silver species partitioning in OECD medium containing Suwannee River humic acid (5 and 50 mg/l) performed using Visual Minteq v3.0 software (J.P. Gustafsson, Stockholm, 2011). Speciation modelling was performed using a fixed concentration of silver (10 µg/l) and concentrations of OECD medium salts provided in Appendix 1. HAAG\textsubscript{(aq)} denotes a soluble monodentate complex of humic acid and silver, whereas HA-Ag\textsuperscript{+}G\textsubscript{(aq)} denotes weakly electrostatically bound soluble complexes of humic acid and silver. Speciation expressed as a percentage of total silver (99.99 % and 100.1% of total silver accounted for in 5 and 50 mg/l SRHA respectively, due to rounding errors).

<table>
<thead>
<tr>
<th>Ag species</th>
<th>5 mg/l SRHA</th>
<th>50 mg/l SRHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag\textsuperscript{+}</td>
<td>42.40</td>
<td>35.85</td>
</tr>
<tr>
<td>AgCl\textsubscript{(aq)}</td>
<td>52.90</td>
<td>44.87</td>
</tr>
<tr>
<td>AgCl\textsubscript{2-}</td>
<td>3.02</td>
<td>2.56</td>
</tr>
<tr>
<td>AgSO\textsubscript{4-}</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>AgNH\textsubscript{3+}</td>
<td>0.58</td>
<td>0.06</td>
</tr>
<tr>
<td>Ag(NH\textsubscript{3})\textsubscript{2+}</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td>HA-Ag\textsuperscript{+}G\textsubscript{(aq)}</td>
<td>0.00</td>
<td>0.09</td>
</tr>
<tr>
<td>HAAG\textsubscript{(aq)}</td>
<td>1.02</td>
<td>16.54</td>
</tr>
</tbody>
</table>

Table 3.11: Dissolved silver species partitioning in JM containing Suwannee River humic acid (5 and 50 mg/l) performed using Visual Minteq v3.0 software (J.P. Gustafsson, Stockholm, 2011). Speciation modelling was performed using a fixed concentration of silver (10 µg/l) and concentrations of JM salts provided in Appendix 1. HAAG\textsubscript{(aq)} denotes a soluble monodentate complex of humic acid and silver, whereas HA-Ag\textsuperscript{+}G\textsubscript{(aq)} denotes weakly electrostatically bound soluble complexes of humic acid and silver. Speciation expressed as a percentage of total silver (100 % and 100.1% of total silver accounted for in 5 and 50 mg/l SRHA respectively, due to rounding errors).

<table>
<thead>
<tr>
<th>Ag species</th>
<th>5 mg/l SRHA</th>
<th>50 mg/l SRHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag\textsuperscript{+}</td>
<td>94.99</td>
<td>76.56</td>
</tr>
<tr>
<td>AgCl\textsubscript{(aq)}</td>
<td>2.47</td>
<td>2.00</td>
</tr>
<tr>
<td>AgSO\textsubscript{4-}</td>
<td>0.28</td>
<td>0.23</td>
</tr>
<tr>
<td>AgNO\textsubscript{3(aq)}</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>HA-Ag\textsuperscript{+}G\textsubscript{(aq)}</td>
<td>0.02</td>
<td>0.17</td>
</tr>
<tr>
<td>HAAG\textsubscript{(aq)}</td>
<td>2.17</td>
<td>20.99</td>
</tr>
</tbody>
</table>
MOPS buffered media had a greater effect on changes in silver speciation at pH 6 compared to pH 8 in both media (Table 3.12 and 3.13). pH 6 reduced the proportion of Ag\(^+\) relative to unbuffered OECD medium (42.62 % to 33.75 %), and increased the proportion of AgCl\(_{aq}\) (53.17 % to 61.11 %) (Table 3.6 cf. Table 3.12). pH 8 had much less of an effect on silver speciation, but slightly increased free Ag\(^+\) (42.62 % to 44.76 %), and decreased AgCl\(_{aq}\) (53.17% to 51.38%) relative to unbuffered OECD medium. A similar effect was observed in JM, with buffering to pH 6 decreasing Ag\(^+\) (97.11 % to 78.21 %) and increasing AgCl\(_{aq}\) (2.53 % to 21.23 %) relative to unbuffered JM (Table 3.7 cf. 3.13). JM buffered to pH 8 changed Ag\(^+\) speciation by < 0.5 % compared to unbuffered JM (Table 3.7 cf. 3.13).

**Table 3.12:** Dissolved silver species partitioning in OECD medium containing 3.5 mM 3-(N-morpholino)propanesulfonic acid (buffered to pH 6 and 8) performed using Visual Minteq v3.0 software (J.P. Gustafsson, Stockholm, 2011). Speciation modelling was performed using a fixed concentration of silver (10 µg/l) and concentrations of OECD medium salts provided in Appendix 1. Speciation expressed as a percentage of total silver (99.99 % and 100 % of total silver accounted for in pH 6 and pH 8 medium respectively, due to rounding errors).

<table>
<thead>
<tr>
<th>Ag species</th>
<th>pH 6</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag(^+)</td>
<td>33.75</td>
<td>43.76</td>
</tr>
<tr>
<td>AgCl(_{aq})</td>
<td>61.11</td>
<td>51.38</td>
</tr>
<tr>
<td>AgCl(^-)</td>
<td>5.09</td>
<td>2.93</td>
</tr>
<tr>
<td>AgSO(_4)^-</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>AgNH(_3)^+</td>
<td>0.01</td>
<td>1.64</td>
</tr>
<tr>
<td>Ag(NH(_3))(_2)^+</td>
<td>0.00</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table 3.13: Dissolved silver species partitioning in JM containing 3.5 mM 3-(N-morpholino)propanesulfonic acid (buffered to pH 6 and 8) performed using Visual Minteq v3.0 software (J.P. Gustafsson, Stockholm, 2011). Speciation modelling was performed using a fixed concentration of silver (10 µg/l) and concentrations of JM salts provided in Appendix 1. Speciation expressed as a percentage of total silver (100 % of total silver accounted for in both pH 6 and pH 8 medium).

<table>
<thead>
<tr>
<th>Ag species</th>
<th>pH 6</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag⁺</td>
<td>78.21</td>
<td>97.25</td>
</tr>
<tr>
<td>AgCl(aq)</td>
<td>21.23</td>
<td>2.40</td>
</tr>
<tr>
<td>AgCl₂⁻</td>
<td>0.27</td>
<td>0.26</td>
</tr>
<tr>
<td>AgSO₄⁻</td>
<td>0.23</td>
<td>0.02</td>
</tr>
<tr>
<td>AgNO₃(aq)</td>
<td>0.06</td>
<td>0.07</td>
</tr>
</tbody>
</table>

3.5.4. Summary of comparisons between media

*Behaviour of NM300K and AgNO₃ concentration response studies*

For NM300K, on a total silver mass basis, the EC₅₀ value was significantly higher in OECD medium compared to JM at 48 (W = 113, p = 0.01) and 72 (F = 19.56, p < 0.001) hours, but not at 24 hours, indicating lower toxicity in the standard medium at these later timepoints. In addition, the EC₅₀ value was significantly reduced over time in both OECD medium (H = 11.39, p = 0.003) (Table 3.1) and JM (F = 6.48, p = 0.006) (Table 3.4), indicating that NM300K toxicity declines over time. The EC₅₀ value was also significantly higher (F = 6.08, p = 0.017) in OECD medium compared to JM, when expressed as ICP-MS mean dissolved silver from NM300K (Section 2.5.1.), but only at 72 hours (F = 9.48, p = 0.007), indicating a similar lower toxicity in the standard medium. Similarly to other silver concentration-response studies, EC₅₀ values, expressed as mean dissolved silver, significantly increased over time in both media (F = 10.97, p < 0.001) (Table 3.13). Finally, when NM300K concentration-response data was expressed on the basis of predicted Ag⁺ only, (Table 3.6 and Table 3.7), the EC₅₀ value was significantly lower in OECD medium compared to JM at 24 (W = 47, p < 0.001) and 72 (F = 7.76, p < 0.013) hours, but not at 48 hours, indicating a higher toxicity in the standard medium at these timepoints (Table 3.8). Given these results it is clear that the toxicity of NM300K changes depending on which concentration metric is expressed (i.e. total silver, dissolved silver, Ag⁺ ions) and in which medium tests are carried out in.

Conversely for AgNO₃, the EC₅₀ value was significantly higher in JM compared to OECD medium at 24 (F = 11.12, p = 0.029) and 48 (F = 93.38, p = 0.001) hours, but not at 72 hours,
indicating lower toxicity in the alternative medium at these timepoints. As with NM300K, the EC₅₀ value of AgNO₃ significantly increased over time in both OECD medium (H = 7.20, p = 0.027) (Table 3.2) and JM (H = 6.49, p = 0.039) (Table 3.4). When AgNO₃ concentration-response data was expressed on the predicted Ag⁺ only (Table 3.6 and Table 3.7), the EC₅₀ value was significantly lower in OECD medium compared to JM at 24 (F = 26.90, p = 0.007), 48 (F = 188.77, p < 0.001) and 72 (F = 271.03, p < 0.001) hours, indicating a higher toxicity in the standard medium at all timepoints (Table 3.9).

**Behaviour of NM300K and AgNO₃ in humic acid studies**

In humic acid (and pH studies), the chosen silver concentration exposures (190 µg/l NM300K and 6 µg/l AgNO₃ in OECD medium; 80 µg/l NM300K and 11 µg/l AgNO₃ in JM) resulted in differences in observed growth inhibition which prevented direct quantitative comparisons between media. Despite this general patterns of humic/pH effect were observed. In toxicant free control cultures, *R. subcapitata* growth rate was slightly reduced with increasing SRHA concentration, though this effect was more evident in OECD medium (Figure 3.5) than in JM (Figure 3.14). In both OECD medium and JM, SRHA addition reduced both NM300K and AgNO₃ growth inhibition in a concentration dependent manner (Table 3.14), with the greatest reduction in toxicity observed at 50mg/l SRHA in all experiments. Whilst NM300K and AgNO₃ toxicity typically decreased (i.e. reduced growth inhibition) from 24 to 72 hours, this effect was not as strong in either media containing 50 mg/l SRHA (Figure 3.5, Figure 3.6, Figure 3.15 and Figure 3.16). When the effect of humic acid on dissolved silver speciation was modelled in both media, a concentration dependent increase in relative percentage of silver-humic acid complexes was observed in both OECD medium (Table 3.10) and JM (Table 3.11). In both media, this effect was accompanied by a relative decrease in Ag⁺, and to a lesser extent AgCl(aq) (Table 3.10 and Table 3.11).
Table 3.14: 72 hour growth inhibition of NM300K and AgNO₃ to *R. subcapitata* in OECD medium and JM, containing 0, 5 or 50 mg/l Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls). Standard error of the mean is shown in parentheses (n_{NM300K} = 3; n_{AgNO₃} = 1, one experiment with three replicates). Superscript letters denote significant differences in growth inhibition within medium/toxicant, between humic acid level (OECD AgNO₃ and JM NM300K - One way ANOVA; OECD NM300K - Kruskal-Wallis; p < 0.05); growth inhibition values not sharing the same superscript letter were significantly different.

<table>
<thead>
<tr>
<th></th>
<th>Growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/l SRHA</td>
</tr>
<tr>
<td>OECD NM300K (190 µg/l Ag)</td>
<td>111.63 (±5.56)⁴ %</td>
</tr>
<tr>
<td></td>
<td>39.76 (±7.45)⁴AB %</td>
</tr>
<tr>
<td>OECD AgNO₃ (6 µg/l Ag)</td>
<td></td>
</tr>
<tr>
<td>JM NM300K (80 µg/l Ag)</td>
<td>82.37 (±5.34)⁴B %</td>
</tr>
<tr>
<td></td>
<td>154.78 (±2.03) %</td>
</tr>
<tr>
<td>JM AgNO₃ (11 µg/l Ag)</td>
<td></td>
</tr>
</tbody>
</table>

**Behaviour of NM300K and AgNO₃ in pH studies**

In pH studies, a greater difference between media were observed. In toxicant free control cultures, *R. subcapitata* growth rate was less effected by pH modification in both OECD (Figure 3.9) and JM (Figure 3.19), compared with humic acid studies (Figure 3.5 and Figure 3.14). In silver growth inhibition studies in OECD medium, pH 6 medium had no significant effect on 72 hour growth inhibition relative to unbuffered medium, for 190 µg/l NM300K or 6 µg/l AgNO₃. At pH 8 however 72 hour growth inhibition was significantly higher for both forms of silver (Table 3.15). Whilst pH induced patterns of toxicity for NM300K and AgNO₃ in JM (Figure 3.20 and Figure 3.21) appeared similar to those observed in OECD medium (Figure 3.10 and 3.11), fewer of the differences in toxicity as a result of pH were found to be significant. A similar change in silver speciation was predicted in both media as a result of pH. Media buffered to pH 6 decreased relative percentages of Ag⁺ and increased AgCl_{aq} in both OECD medium (Table 3.6 and Table 3.12) and JM (Table 3.7 and Table 3.13). In media buffered to pH 8 the opposite effect was observed, an increase in relative percentage of Ag⁺ and a decrease in AgCl_{aq} in both OECD medium (Table 3.6 and Table 3.12) and JM (Table 3.7 and Table 3.13).
Table 3.15: 72 hour growth inhibition of NM300K and AgNO₃ to *R. subcapitata* in OECD medium and JM, in unbuffered medium, medium buffered to pH 6 or 8 (buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid). Data expressed as mean percentage growth inhibition (compared to toxicant free controls). Standard error of the mean is shown in parentheses (NM300K = 3; AgNO₃ = 1, one experiment with three replicates). Superscript letters denote significant differences in growth inhibition within medium/toxicant, between pH level (One way ANOVA; p < 0.05); growth inhibition values not sharing the same superscript letter were significantly different.

<table>
<thead>
<tr>
<th></th>
<th>Growth inhibition (%)</th>
<th>pH 6</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OECD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM300K (190 µg/l Ag)</td>
<td>59.80 (±11.17)⁸ %</td>
<td>84.69 (±21.53)⁸ %</td>
<td>123.41 (±13.15)⁸ %</td>
</tr>
<tr>
<td>AgNO₃ (6 µg/l Ag)</td>
<td>34.59 (±4.97)⁸ %</td>
<td>49.56 (±2.95)⁸ %</td>
<td>94.10 (±5.79)⁸ %</td>
</tr>
<tr>
<td><strong>JM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM300K (80 µg/l Ag)</td>
<td>38.22 (±8.81)⁸ %</td>
<td>14.90 (±5.07)⁸ %</td>
<td>69.78 (±7.75)⁸ %</td>
</tr>
<tr>
<td>AgNO₃ (11 µg/l Ag)</td>
<td>124.14 (±4.50)⁸ %</td>
<td>116.21 (±2.32)⁸ %</td>
<td>117.13 (±2.62)⁸ %</td>
</tr>
</tbody>
</table>

3.6. Discussion

3.6.1. Patterns of toxicity in unmodified media

Both NM300K and AgNO₃ induced a concentration dependent inhibition of *R. subcapitata* growth rate, of exposure in all unmodified media (i.e. unbuffered and SRHA free) studies. The toxicity of silver (ionic and particulate) decreased over time, as indicated by higher EC₅₀ values at 72 hours. As hypothesised, AgNO₃ exhibited higher overall toxicity than NM300K in both media. When calculated on the basis of mean ICP-MS derived dissolved silver only the toxicity of NM300K was still lower in OECD medium compared to JM, though the difference in toxicity was less than when expressed on a total silver mass basis. In addition, differences in NM300K and AgNO₃ toxicity were observed between OECD and JM media. On a total silver mass basis, NM300K toxicity was lower (i.e. higher EC₅₀) in OECD medium compared to JM. Finally, as some authors have suggested that silver is taken up by algae as Ag⁺, and this species is the primary determinant silver toxicity (Lee *et al.*, 2004; Zhang *et al.*, 2012; Mousavi *et al.*, 2015), silver speciation was predicted for both media, using ICP-MS derived dissolved silver (Section 2.5.1.1.). In JM, the majority of total dissolved silver was predicted to be in the form of free ions (97.11 % Ag⁺), with a small proportion of silver chloride (2.53 % AgCl(aq)). In OECD medium however, only 42.62 % of total dissolved silver was predicted to be in the Ag⁺ form, with 53.17 % as AgCl(aq). Given that Cl⁻ has been demonstrated to reduce silver toxicity by binding to free Ag⁺ (Lee *et al.*, 2004), such findings support the observed reduction in toxicity of NM300K in OECD medium compared to JM. This finding is further supported by the fact that OECD medium has more chloride than JM; 22.99 mg/l Cl compared to 0.5 mg/l Cl (Appendix 1).
Indeed, when EC_{50} values were recalculated on the basis of modelled Ag^{+} concentration, NM300K toxicity became slightly but significantly higher in OECD medium compared to JM. In summary, OECD medium appeared to reduce the toxicity of NM300K relative to JM, and such effects are likely attributable to a combination of lower dissolution (as measured by ICP-MS, Section 2.5.1.) and a higher degree of AgCl_{aq} complexation. Based on the findings presented here, the hypothesis that silver speciation (as a result of medium composition) may be the principle contributing factor in toxicity estimates for silver nanoparticles can be tentatively accepted. However estimates of both total dissolved silver (derived from algae-free samples using ICP-MS; Section 2.5.1.1.) and dissolved silver speciation (modelled using Visual Minteq; Section 3.5.3.) have considerable limitations, which limit the ability to generalise their findings to the dynamic nature of actively growing algal cultures. Such hypotheses require further investigation using experimental methods which can distinguish between dissolved silver speciation in real time exposures (unlike ICP-MS/Visual Minteq), such as ion selective electrodes.

For ionic silver (AgNO_{3}), when exposure concentration was quantified on a mass basis, the toxicity was greater than that of NM300K in both media. Unlike NM300K, on a total silver mass basis AgNO_{3} was more toxic in OECD medium than in JM. However once silver speciation was taken into account in both media this pattern of toxicity was retained. As described previously for NM300K, observed toxicity may not be described best by total silver mass, rather by resulting speciation of total dissolved silver.

Data generated in this work are comparable to the existing literature on the toxicity of both ionic and nanoparticulate silver to algae (Bondarenko et al., 2012; Table 3.16). Ionic silver has an EC_{50} value in algae in the range of 1.5-600 µg/l, depending on experimental conditions (e.g. species, exposure time, medium composition, and endpoint) (see Bondarenko et al., 2012 for review). As observed in the work presented here, silver nanomaterials have a higher algal EC_{50} value relative to ionic silver, in the range of 3-20,000 µg/l (see Bondarenko et al., 2012 for review). This observed silver nanomaterial EC_{50} range is also affected by the same experimental conditions as for ionic silver, in addition to dispersion method and nanomaterial characteristics. The reported variation in toxicity for silver nanoparticles is higher than that reported for soluble silver salts. Such differences are likely to derive from the use of silver nanoparticles of varied physico-chemical properties (such as particle coating, size, shape) in existing studies, the use of different preparation methods to disperse nanoparticles (e.g. sonication), the influence of ageing (e.g. dissolution, agglomeration and aggregation during storage), methodology used to assess toxicity (e.g. medium selection, time point investigated, species tested, cell density). As a result stating toxic properties of “silver nanoparticles” in
general has very little meaning without detailed descriptions of characterisation and experimental design.

Using the same standard experimental design (OECD 201 test, OECD medium, extracted Chl a quantification) with *Chlorella vulgaris*, Kalman et al. (2015) found a mean 72 hour EC50 value of 9.3 (±0.1 s.d.), 9.2 (±1.0 s.d.) and 49.3 (±5.2 s.d.) µg/l Ag for polyvinylpyrrolidone- (PVP), citrate- and polyethylene glycol- (PEG) coated silver nanoparticles (10 nm), respectively. In the same study, 72 hour EC50 value for AgNO3 was 5.3 (±0.5 s.d) µg/l Ag, indicating that *C. vulgaris* shows a similar sensitivity to ionic silver as *R. subcapitata*. Ribeiro et al. (2014) found a lower toxicity of AgNO3 to *R. subcapitata* (72 hour EC50 = 33.79 ±2.96 s.e. µg/l Ag), however a difference in experimental design may have affected toxicity estimates (e.g. initial cell density, test vessels, photoperiod, enumeration method). Few studies have specifically assessed the toxicity of NM300K to *R. subcapitata* (Wang et al., 2012; Sorensen and Baun, 2015). Sorensen and Baun (2015) found that in OECD medium, NM300K and AgNO3 caused a 48 hour growth inhibition (EC50) of 140 (120-160; 95 % confidence) and 4.9 (3.9-6.2; 95 % confidence) µg/l Ag, respectively. In the work presented in this chapter, at comparable timepoints, the 48 hour EC50 value was lower (80.33 ±2.40 and 2.22 ±0.06 µg/l Ag for NM300K and AgNO3) indicating higher toxicity. However the test design in Sorensen and Baun’s (2015) work was in a miniaturised version of the standard OECD test (Arensberg et al., 1995), which may have accounted for their lower sensitivity observed (discussed in detail in Chapter 4). In addition, it is worth noting that from a risk assessment perspective, this difference in toxicity observed between studies is not particularly large.

Wang et al. (2012) found a 4.5 hour photosynthetic inhibition EC50 value of 928.74 (860.79-968.65; 95 % confidence) µg/l Ag for NM300K; however photosynthetic inhibition is a less sensitive endpoint than growth inhibition (Navarro et al., 2008; Chapter 5 in this thesis). Thus, assessment of nanomaterial 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, especially when using non-standard endpoints/test systems, to serve as a benchmark for comparison between different studies. Most studies investigating nanoparticles and algae also investigated the toxicity of AgNO3 in their test systems (Navarro et al., 2008; Wang et al., 2012; Ribeiro et al., 2014; Kalman et al., 2015; Sorensen and Baun, 2015). The inclusion of ionic controls in nanoparticle toxicity studies of this nature should be considered as best practice; due to the highly variable properties of nanoparticles and potential artefacts associated with 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 nanomaterial toxicity studies.

### 3.6.2. Differences between media

A number of studies have found that medium composition can significantly affect the behaviour of silver nanoparticles and their resulting toxicity to environmental model organisms such as *Daphnia magna* (Shen et al., 2015) and cell (e.g. isolated from *Oncorhynchus mykiss* gills) (Yue et al., 2015). The two media used in this chapter, OECD medium and JM, are considerably different in their composition. JM has a higher ionic strength than OECD medium due to higher concentrations of both major and minor elements (Appendix 1). Such higher concentrations of these elements are likely to provide a higher supply of nutrients which sustain optimal algal growth for longer periods (i.e. 72 hour growth rate of control cultures of *R. subcapitata* were slightly but significantly higher in JM). In addition, the primary nitrogen source of JM is NO$_3^-$, which causes the pH to rise over the duration of the test in actively growing controls. As mentioned previously, a similar change in control cultures is not observed for OECD medium due to the presence of NH$_4^+$ as the primary nitrogen source, which releases protons during nitrogen assimilation and offsets increases in pH during active growth (Arensberg et al., 1995; Aruoja, 2011). Considering the logarithmic scale of pH, the permitted $< 1.5$ unit change of the duration of a standard OECD 201 test (OECD, 2011) represents a considerable pH drift, especially given that the data presented here indicate significant differences in ionic silver and nanoparticle toxicity can be induced through a maximum test medium pH difference of 2 units. The presence of various salts ubiquitous in both of the media studied here (CaCl$_2$, MgSO$_4$, and NaHCO$_3$) have been demonstrated to affect silver nanoparticle aggregation/agglomeration, and resultant speciation through reactions of Ag$^+$ with Cl$^-$, SO$_4^{2-}$, and CO$_3^{2-}$ (Roemer et al., 2011; Tejamaya et al., 2012; Seo et al., 2014). Whilst such physical effects of nanoparticle interactions are usually the focus of such studies, the chemical speciation of dissolved silver in different ecotoxicological media remains unclear. As a result, whilst the OECD 201 test does not place require strict constraints upon media choice or pH control, it is the use of standard OECD medium should be considered best practice in future testing. When alternative media use is unavoidable, pH drift should be kept to a minimum to avoid unnecessary variation in exposure conditions (for further discussion of pH control, see Section 3.6.4.).

On a total silver mass basis, NM300K was 2.4 times more toxic to *R. subcapitata* in JM than OECD medium. There is a large difference in chloride concentration between the two
media, with OECD medium containing and JM (Appendix 1). A clear difference in silver speciation was predicted using Visual Minteq between both test media, especially in relation to Ag⁺. In OECD medium, only 42.62 % of silver is Ag⁺, with 52.17 % bound as AgCl(aq). However the same treatment in JM, 97.11 % of silver is in the form Ag⁺, with only 2.53 % bound as AgCl(aq). In both media AgSO₄ was a minor species, as were nitrogenous silver species (AgNH₃⁺, Ag(NH₃)₂⁺ and AgNO₃(aq)). Lee et al. (2004; 2005) showed that the presence of chloride significantly reduced the uptake and resultant toxicity of ionic silver to R. subcapitata, which exhibits “slow” silver uptake rates (i.e. Ag⁺ is a primary predictor of uptake and toxicity, slow uptake rates prevent diffusion limitation under BLM). When NM300K EC₅₀ values were recalculated on the basis of both dissolved and subsequent Ag⁺ partitioning in each medium (JM and OECD), the difference in toxicity between media was no longer as pronounced. This suggests that the presence of chloride in OECD medium may be responsible for the reduced toxicity of NM300K observed in this media, and that silver nanoparticle toxicity is mainly driven by dissolved silver (in particular free Ag⁺). Wang et al. (2012) found NM300K contained 1-10 % Ag⁺ in an unspecified medium (total dissolved silver was not measured), and that ~35-80 % of its toxic effect on R. subcapitata could be attributed to free Ag⁺. Post hoc calculations can therefore be of some use to explain differences in observed toxicity of NM300K in different media, however more detailed study would be needed to fully investigate these phenomena. Such work could include quantification of silver losses to the test system (Lee et al., 2005), algae-medium interactions (Koukal et al., 2007; Miao et al., 2009), and direct real time measurement of free Ag⁺ using ion selective electrodes (Navarro et al., 2008; Gonsolus et al., 2015; Mousavi et al., 2015; Shen et al., 2015). Furthermore ICP-MS data for NM300K could be improved through the inclusion of AgNO₃ controls, which may give some indication of silver losses to the test system/sample preparation procedure.

As demonstrated here, algal growth medium choice can have significant effects on altering silver toxicity. The OECD 201 test guideline recommends either OECD or the Algal Assay Procedure (AAP) medium, but allows for the use of alternative media, providing such usage is well justified and described (OECD, 2011). As such differences in media make such a choice non-trivial, it is important that adequate consideration is given to medium selection in terms of comparability with existing literature and regulatory documents. As the OECD 201 test is one of the suggested mandatory ecotoxicological tests required for REACH regulations at the one tonne production level (EU Directive, 1999/45/EC), it is important that media choice remains comparable between applications so as not to undermine the value of an important risk assessment database. It is therefore recommended that the standard OECD media be used where possible, or deviations from it be well described and justified.
3.6.3. Influence of humic acid on silver toxicity

Results presented here demonstrate that SRHA reduced the toxicity of ionic and nanosilver toxicity in a concentration dependent manner, with the greatest effect observed at higher concentrations of SRHA (50 mg/l). This was observed at almost every timepoint studied, and with all combinations of silver and test medium, generally supporting the hypothesis that humic acid would reduce silver toxicity. Exceptions to these observations were for AgNO₃ at 72 hours in OECD medium, where silver exerted a low toxic effect in all three humic acid conditions.

The presence of humic substances has been demonstrated to reduce silver (nanoparticle and ionic) toxicity in a number of environmental organisms, such as bacteria (Fabrega et al., 2009; Zhang et al., 2012; Gunsolus et al., 2015; Mousavi et al., 2015), algae (McLaughlin and Bonzongo, 2012), daphnids (Gao et al., 2012; McLaughlin and Bonzongo, 2012) and Oryzias latipes (Kim et al., 2013). Chen et al. (2013) demonstrated that SRHA concentrations up to 10 mg/l had no significant effect on ionic silver-induced growth inhibition in C. reinhardtii at 24 hours; although the authors hypothesised that this effect may increase over longer test durations, as demonstrated in the work presented here. More specifically, in the results presented here, a concentration of 5 mg/l SRHA had little effect in reducing the toxicity of silver (both ionic and nanoparticulate) in either medium. However, at higher SRHA concentrations (50 mg/l), a significant reduction of growth inhibition was observed for both forms of silver, in both media, and at every timepoint. The only exception to this was in OECD medium containing 6 µg/l AgNO₃, at 72 hours, and was likely due to the fact that exposure of algae to AgNO₃ in the absence of SRHA did not exert a sufficiently strong toxic effect to be mitigated by SRHA. Interestingly, a concentration of 50 mg/l SRHA also stabilised toxicity over time, resulting in similar levels of silver induced growth inhibition at every timepoint which was not observed in any other medium condition.

Due to both the complex chemical nature of humic substance (including SHRA), and uncertainty around ionic/nanoparticle silver mode of toxicity, a number of mechanisms by which humic substances mitigate toxicity have been proposed. Some studies have found humic substances increase the stability of unstable nanomaterial suspensions (unlike NM300K) by binding to the particle surface, preventing aggregation/agglomeration (Gondikas et al., 2015) and possibly reducing Ag⁺ release (Yang et al., 2014). However the release of Ag⁺ from nanomaterials is relatively insensitive to aggregation/agglomeration state (Zhang et al., 2011; Liu and Hurt, 2012; Gondikas et al., 2015). NM300K used in this study is considered to be relatively stable in the absence of humic substances, due to steric stabilisation with dispersants.
(polyoxyethylene (20) sorbitan monolaurat and polyoxyethylene glycerol trioleate; Klein et al., 2011). Steric stability of nanomaterials cannot be effectively detected using DLS (which is more suited to detecting electrostatic stabilisation), and SRHA had little effect on electrostatic stabilisation, especially in OECD medium (Section 2.5.2.). Humic substances have been hypothesised to be less effective at conferring additional stability on sterically stable silver nanoparticles, due to such dispersants having a higher affinity for the nanoparticle surface the humic substances (Gondikas et al., 2015). As SRHA reduced toxicity of both NM300K and AgNO₃ in the same manner, such observations indicate a likelihood of SRHA interacting with dissolved silver speciation in the media studied.

The extent to which humic substances affect the dissolution of silver nanoparticles varies between studies. Pokhrel et al. (2013; 2014) found that humic substances increased silver nanoparticle dissolution, whereas other studies have found decreased dissolution (Liu and Hurt, 2010; Zhang et al., 2012). Whilst these studies did not distinguish between different species of dissolved silver (including Ag⁺ complexation with humic substances), such differences have been attributed to differing methodologies (Gondikas et al., 2015). A number of studies have attributed the reduction in silver nanoparticle toxicity to the complexation of humic substances with Ag⁺ (Fabrega et al., 2009; Wirth et al., 2012; Zhang et al., 2012; Yang et al., 2014; Gondikas et al., 2015). Whilst SRHA appeared to result in an increase in total dissolved silver in both media (Section 2.5.1.), speciation modelling revealed a concentration dependant reduction in Ag⁺ in both OECD medium and JM in the presence of SRHA. If Ag⁺ is the silver species which primarily contributes to toxicity, these findings further support this hypothesis.

In both OECD medium and JM, silver (ionic and nanoparticle) toxicity decreased over the test duration (see Section 3.5.6. for further detail). However in the presence of 50 mg/l SHRA, the same time dependent decrease in toxicity was not observed in either form of silver, in both media. Whilst 50 mg/l SRHA reduced toxicity of both ionic and nanoparticulate silver relative to humic free and 5 mg/l SRHA, it did not induce the typical time-dependent toxicity decrease as observed in other test conditions. Mousavi et al. (2015) found that in pH 7.5 buffered, chloride-free media of fixed ionic strength, 50 mg/l SRHA reduced 15.4 % of total Ag⁺ (539.3 µg/l), within one second of mixing, using an ion selective electrode with high temporal resolution. When predicting dissolved silver speciation, a similar complexation proportion of silver was predicted in this work, suggesting such an effect is relatively insensitive to media composition. When silver-humic complexes form, Ag⁺ is likely bound to compounds rich in nitrogen, sulphur, and to a lesser extent oxygen (Sikora and Stevenson, 1988), not to mention compounds of poorly understood chemical composition (Maurer-Jones
et al., 2013). Whilst some of these compounds will undoubtedly be responsible for the observed decrease in toxicity as a result of the formation of silver-humic complexes (McLaughlin and Bonzongo, 2012; Zhang et al., 2012; Kim et al., 2013), the rapid binding of silver to SRHA (Mousavi et al., 2015) may compete with other, slower acting ‘sinks’ of silver (i.e. factors causing loss of silver to the test system such as glassware or algal cells themselves; Chen et al., 2013; Sekine et al., 2015) which would otherwise remove it from the test system, resulting in the observed decrease in toxicity over time. Indeed, SRHA not only reduced Ag⁺ in the media studied here, but also to a lesser extent reduced proportions of competing species such AgCl(aq) AgNO₃(aq) AgNH₄⁺. SRHA has also been found to stabilise aggregating silver nanoparticles (Gunsolus et al., 2015), but these results suggest a broader stabilising effect on silver and its resulting toxicity to algae, possibly by the presence of bioavailable sulphurous compounds (Fortin and Campbell, 2001; Hiriart-Baer et al., 2006). This competitive speciation and resulting stability may result in a net decrease in toxicity, but have important implications for the persistence of silver-humic complexes in the environment.

If BLM is a suitable model of predicting silver-algal interactions, and toxicity is driven by Ag⁺, the characterisation and toxicity data presented here support the argument that quantification of total dissolved silver (compared to Ag⁺) is a poor predictor of toxicity in R. subcapitata (Lee et al., 2004; 2005). By contrast some authors have observed adherence of Ag-SRHA complexes to algal cells, which had no influence on toxicity compared to humic acid free exposures, which does not support BLM (Chen et al., 2013). In addition, there are obvious implications for higher trophic level effects through ingestion by primary consumers (Fabrega et al., 2011). High concentrations of humic acid caused a predicted higher reduction in Ag⁺, relative to SRHA-free media, in JM compared to OECD medium. This may explain the larger decrease in toxicity in JM, at comparable concentrations of NM300K. Such conclusions are not possible to make for AgNO₃, as toxicant effect sizes between media are not comparable (i.e. there was a smaller effect of the chosen AgNO₃ concentration on toxicity in OECD medium).

3.6.4. Influence of pH on silver toxicity

Compared to the humic acid results, patterns in silver toxicity as a result of pH varied more greatly between silver form and test medium. Silver toxicity was generally unchanged when comparing results in unbuffered test medium to medium buffered to pH 6. However in studies of NM300K in JM, silver toxicity in pH 6 medium was approximately half that observed in unbuffered medium, at all timepoints investigated. In media buffered to pH 8, silver toxicity was generally higher than equivalent concentrations in unbuffered medium, an effect which was more pronounced at earlier timepoints. The exception to this observation was in JM
containing AgNO₃, where little difference in silver toxicity was observed between any pH condition investigated. Furthermore, the hypothesis that lower pH would result in higher toxicity could not be accepted; under the experimental conditions investigated in this chapter, a higher pH generally results in greater silver toxicity to *R. subcapitata*.

OECD 201 test guideline allows a pH change of < 1.5 units over the 72 hour duration of a standard test (OECD, 2011), which is a large change in test conditions considering that the pH scale is logarithmic. In order to investigate whether a fixed pH within the range of 1.5 units of OECD test medium (pH 7.5) would affect the test results, MOPS buffer was used to study silver toxicity at pH 6 and 8. Rendal *et al.* (2012) identified an EC₅₀ value for MOPS on *R. subcapitata* growth as 7.3 mM (3.2-16; 95% confidence) and 11 mM (4.6-27; 95% confidence) over 48 and 72 hours, respectively. According to OECD 201 test guideline (OECD, 2011), MOPS is adequate to prevent pH drifts of ≤ 1.5 units over the test duration, at concentrations which are non-toxic to *R. subcapitata*. The data obtained in this study support the use of MOPS, and also demonstrate the suitability of 3.5 mM MOPS as a buffer to maintain a pH of 6 or 8 over the standard 72 hour test duration, even in non-standard media which lack intrinsic buffering capacity (i.e. JM). It is worth noting that buffering capacity of MOPS was not as efficient at pH 6 as at pH 8. This is likely attributable to the fact that pH 6 is slightly outside the pH 6.5-8 optimal buffering range of MOPS.

Although Kandegedara and Rorabacher (1999) reported MOPS to be completely non-complexing with metals, Mousavi *et al.* (2015) demonstrated that MOPS may complex with silver. In OECD medium buffered to pH 6, there was little effect on silver toxicity compared with unbuffered medium. However in the same media buffered to pH 8, silver induced growth inhibition was significantly higher than in unbuffered medium. More specifically, silver toxicity was approximately twice as high, at almost every timepoint at pH 8. In JM, a different pattern of toxicity was observed. When compared to unbuffered JM, the growth inhibition of NM300K at pH 6 was significantly reduced (by approximately half) at 72 hours, but this pH had no significant effect on toxicity at other timepoints. For AgNO₃ pH 6 had no significant effect at any timepoint. In JM at pH 8 silver toxicity was significantly higher than unbuffered medium at earlier timepoints. However this effect was not as pronounced as was observed in OECD medium at the same pH. A possible explanation for the difference between both media at pH 8 could be that the pH of poorly buffered, unmodified JM increased over the duration of the test to the extent that 72 hour pH in unbuffered JM was the same as that of pH 8 buffered JM. These results indicate a more complex relationship than simple Ag⁺-MOPS complexation as described in Mousavi *et al.* (2015). When silver speciation was modelled using Visual Minteq, no complexation with MOPS buffer was observed. Whilst this could indicate that MOPS is out-
competed by other salts in the media for silver, it is more likely that the data supporting Visual Minteq for MOPS has not yet been updated, due to the recency of this complexation discovery (Mousavi et al., 2015). As such, the modelled pH dependent speciation data presented here should be viewed as incomplete.

According to speciation modelling, both MOPS buffered media at pH 8 resulted in a higher relative percentage of Ag\(^+\), and a lower relative percentage of AgCl\(_{\text{aq}}\), than media at pH 6. This may explain why the toxicity of silver is enhanced at a higher pH. Although much research has been carried out demonstrating the effect of increasing pH on increasing algal toxicity for other metals (Macfie et al., 1994; Nalewajko et al., 1997; Franklin et al., 2000; De Schamphelaere et al., 2004), little data exists on the toxicity of both ionic silver and silver nanomaterials to algae as a result of pH changes. Oukarroum et al. (2014) found no difference in patterns of silver nanoparticle toxicity to *Chlamydomonas acidiphila* growth at pH 4 and pH 7; however pH induced differences in toxicity emerged when different endpoints, such cellular viability and ROS production, were chosen. In studies with daphnids, an increase in pH has been demonstrated to reduce toxicity of both AgNO\(_3\) and citrate coated silver nanoparticles (Seitz et al., 2015). Importantly, the pH data presented here shows that the toxicity of silver can vary over a similar pH range as to that permitted over time in the OECD 201 test (i.e. ≤ 1.5 units; OECD, 2011). However this work has also demonstrated that without additional buffering, it is possible to keep a narrow pH range in standard OECD medium control cultures through proper experimental conditions (< 0.1 units, achieved through high vessel shaking speed to increase CO\(_2\) drawdown). In non-standard medium however (e.g. JM), such pH drifts may be more difficult to control and have consequences for resultant toxicity measurements.

The effect of pH 8 on the increased silver toxicity observed in this chapter could also be attributed to indirect synergistic toxic effects. The effect of increased pH on algal physiology may increase their susceptibility to silver, rather than pH having a direct effect on silver speciation and toxicity. One factor which may affect algal susceptibility is the change in carbon availability. An increase in freshwater pH has been established to reduce algal growth due to a reduction in dissolved CO\(_2\), algae’s preferred nutrient source (Azov, 1982). A slight reduction in < 48 hour *R. subcapitata* growth rate was observed in OECD medium (but not JM), which may indicate a degree of synergism, but further experimental work is needed to fully elucidate such an explanation. In addition to carbon availability, a similar explanation may be employed in relation to MOPS. Whilst the concentrations used in this chapter where approximately half the EC\(_{50}\) of MOPS described by Rendall et al. (2012), the effect of this buffer on both algal growth and silver speciation (Mousavi et al., 2015) require further study. Future experiments may use
a range of buffers/buffering methods when studying pH effects on silver toxicity to algae, to identify the potential confounding effect of MOPS and other buffers.

Concentrations of NM300K and AgNO$_3$ were chosen in each media to induce around an 80% growth inhibition of *R. subcapitata*, at 72 hours in order to provide comparable data between studies when investigating the impact of pH and humic acid on silver toxicity. The EC$_{80}$ concentration was identified from concentration response growth inhibition studies. However at the AgNO$_3$ concentrations tested, growth inhibition in JM was higher than predicted, and OECD medium lower than predicted when used to assess the influence of pH and humic acid on silver toxicity. This suggests that the concentration response data for AgNO$_3$ would benefit from testing a narrower concentration range. For NM300K, concentration response relationships spanned a similar effective concentration range in both media, and the 72 hour maximum growth inhibition was closer to the test concentration range in OECD 201 guidelines of 0-80% inhibition (OECD, 2011). Future work may be more successful by adopting a different approach, such as performing duplicate concentration response studies (i.e. the same test concentrations) at different humic acid/pH levels. Such a method was considered in this thesis, but ultimately rejected due to time constraints. Performing a single algal concentration response study in flasks takes 4 days. However by using miniaturised methods such as those presented in Chapter 4, such work can be carried out in parallel with fewer concerns for sample processing time or available space within the test chamber.

### 3.6.5. Role of dissolved and nanoparticulate silver, and applicability of BLM

One of the key questions in nanotoxicology is the extent to which silver nanomaterials themselves, or their associated dissolved ions, contribute to observed toxicity (Lubick *et al.*, 2008; Xiu *et al.*, 2012). Larger differences in toxicity were observed when silver concentrations were expressed on a total silver mass basis between both media and silver form. However when silver concentrations were expressed as a function of ICP-MS derived total dissolved silver (Section 2.5.1.), differences between both media and silver form were greatly reduced. As in other published literature, a higher toxicity of nanoparticles was observed compared to ionic controls, when expressed as total silver (Navarro *et al.*, 2008). Despite this, dissolved silver may exert the dominant effect on algal toxicity, and nanomaterial-specific processes (agglomeration, aggregation, etc.) may modify this toxicity depending on particle characteristics such as ionic release (Sorensen and Baun, 2015). In reality, such phenomena are hard to measure against background variability from a variety of sources, including detoxification by algal exudates (Koukal *et al.*, 2007; Miao *et al.*, 2010), sorption of toxicant to test vessels (Lee *et al.*, 2005; Malysheva *et al.*, 2015; Sekine *et al.*, 2015) and
differing ion release rates from silver nanomaterials under different test conditions (Kittler et al., 2010; Liu and Hurt, 2010; Sorensen and Baun, 2015). Although existing characterisation data (Klein et al., 2011; Wang et al., 2012) suggest NM300K to be a highly stable particle, this stability did not always translate into consistent toxicity data – a factor attributed to rapid changes in NM300K dissolution over short time periods in environmental media (Sorensen and Baun, 2015). Ideally a variety of physico-chemical properties (dissolution, speciation, losses to test system, etc.) should be measured for every single toxicity test – however such procedures are rarely a practical option.

Whilst uptake of both ionic silver and silver nanoparticles was not assessed in this work, it may be of interest in future studies. A limited number of studies have investigate the uptake, or mechanism of uptake of silver nanoparticles by algal cells. Silver nanoparticle internalisation by mammalian (Carlson et al., 2008), bacterial (Bae et al., 2011) and algal (Miao et al., 2010; Piccapietra et al., 2012, Kalman et al., 2015) cells has been observed. However some studies which have investigated the uptake of nanomaterials by algal cells have used washing steps to remove extracellular metal nanomaterials, followed by acid digestion of exposed algal cells and quantification of remaining ‘internal’ metal using AAS or ICP-MS (Wang et al., 2011; Perreault et al., 2012; Piccapietra et al., 2012). However such methods may not fully remove strongly externally bound nanomaterials or distinguish between ionic and nanoparticulate metal and may therefore no accurately quantify nanomaterial uptake (Chen et al., 2013). Other studies have used TEM or SEM, in combination with energy dispersive spectrometry (which enables an elemental analysis to be performed), to identify individual metal nanomaterials within exposed algal cells (Miao et al., 2010; Wang et al., 2011; Perreault et al., 2012; Kalman et al., 2015). TEM alone cannot identify nanomaterial elemental composition (Miao et al., 2010), and nanomaterials can resemble sub-cellular structures (Johnston et al., 2015) calling into question whether observed particles are the ones of interest. Even when TEM is combined with elemental analysis methods, the presence of nanomaterials within the cells may simply be an artefact of sample preparation (Carlson et al., 2008) or natural nanomaterials formed within the cell after uptake of metal ion may be visualised (Zhang et al., 2011). Some authors have proposed that uptake of nanomaterials by algal cells occurs via endocytosis (Miao et al., 2010), or through membrane damage via the creation of pores which allow nanomaterials to enter cells (Navarro et al., 2008; Kalman et al., 2015). More recently, studies using Coherent Raman Anti-stokes Raman Scattering (CARS) microscopy have demonstrated that 100 µm silver nanoparticles are neither internalised by R. subcapitata, nor closely bound to the cell wall, after 48 hours of exposure (Ribeiro et al., 2015). CARS microscopy is an advantageous method when investigating nanomaterial
internalisation, as it does not require nanomaterials to be labelled (e.g. with fluorescence) to enable their imaging, can be used with live cells without staining, reducing the chance of artefacts in sample preparation (Johnston et al., 2015; Ribeiro et al., 2015). Thus future studies could use this imaging modality to better understand the uptake of nanomaterials by algal cells.

Concentrations of both ionic and nanoparticulate silver, in humic acid and pH experiments, were adjusted on the basis of modelled Ag⁺ availability and used to predict toxicity according to the probit models generated in concentration-response experiments. However Ag⁺ induced toxicity proved to be a poor predictor of observed toxicity data in both media. These results suggest that free Ag⁺ may not be best determinant of silver toxicity to R. subcapitata in the presence of complex organic substances, as some authors have suggested (Fortin and Campbell 2001; Campbell et al., 2002; Hiriart-Baer et al., 2006; Chen et al. 2013). However this poor predictive power of Ag⁺ in complex media does not deny the importance of BLM, rather it constrains the applicability of the model to algae in more simple media (i.e. containing inorganic salts such as OECD and JM) (Campbell et al., 2002).

3.6.6. Reduction in toxicity over the test duration

In nearly all test conditions, both ionic and nanosilver showed a time-dependent decrease in toxicity (i.e. higher EC₅₀, or lower effect size at a fixed silver concentration), with the only exception being in media containing 50 mg/l SRHA. This effect is interesting given that NM300K undergoes increased dissolution over 72 hours with the test system (Section 2.5.1.), and so a reduction in toxicity occurs despite an apparent increase in dissolved silver. There are two potential explanations for this observed effect – the test vessels and the test organism. Plastic and glass tests vessels have been shown to adsorb both ionic and nanoparticulate silver (Malysheva et al., 2015; Sekine et al., 2015), with some studies opting for non-standard tests with shorter exposure durations to minimise silver losses (Lee et al., 2004; 2005). Despite such precautionary measures, silver losses were still observed in some cases (Lee et al., 2005). Authors have attributed silver loss to algal exudates (Navarro et al., 2008; Miao et al., 2009), test vessels (Lee et al., 2004; 2005; Chen et al., 2013) or both (Fortin and Campbell, 2001; Hiriart-Baer et al., 2006). In addition dead/lysed cells may remove a portion of silver from the test system by binding irreversibly (in test conditions) to membrane fragments (Chen et al., 2013). The removal of silver from the test medium by algal binding has important implications on reducing the exposure concentrations for surviving cells. If such a hypothesis were true, the implications of this suggest that algae may rapidly detoxify silver in natural environments. From an experimental persepctive, such an effect could be mitigated through the use of
chemostatic exposures (Hiriart-Baer et al., 2006), which could compensate for decreases in silver concentration over the test duration.

Algae have been demonstrated to detoxify metals through the production of phytochelatins and metal-binding polyphosphate granules (Lavoie et al., 2009). Pillai et al. (2014) demonstrated that C. reinhardtii is capable of upregulating an array of biochemical pathways over short term (< 5 hour) silver exposures. Due to the complex nature of “omics” studies of detoxification in algae, most studies to date have investigated in individual combinations of species and toxicant. Despite this, such methods would be of great value in future studies of the interaction of silver with R. subcapitata. One potential detoxification process that has been studied more broadly is exudate production. Algal exudates (also known as extracellular polymeric substances - EPS) are a broad class of dissolved organic matter released by algae and other microorganisms. Polymeric substances have been demonstrated to reduce metal toxicity to algal cells both externally and internally, likely by acting as ligands and altering subcellular metal distribution (Salgado et al., 2005 Haye et al., 2006; Koukal et al., 2007; Miao et al., 2009). R. subcapitata exudates in particular have been found to contain around 50 % carbohydrate and 5 % protein, with total exudate production increasing throughout the exponential growth period of the culture (Koukal et al., 2007). Given the increasing exudate production over time, it is evident that algae in the same exponential phase of growth (as required by OECD 201 test guidelines) may have variable concentrations of associated exudates from previous subculturing history. The OECD 201 test suggests the use of K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} as a standard toxicant and positive control, to regularly check the test procedure (OECD, 2011). However this substance does not appear to exhibit the same time-dependent reduction in toxicity (Appendix 3), and as a result is likely to be insensitive to different exudate detoxification between cultures. Whilst investigating such detoxification hypotheses are beyond the scope of this work, this could be an important source of variation between not only individual tests (as observed here) but also laboratories. Different laboratories have different subculture procedures which may affect exudate production (e.g. preparation of cryogenically preserved algae immediately before testing cf. preparing test cultures from a serially grown culture line). There is currently no provision for such observations in the standard OECD 201 test (OECD, 2011); one potential solution may be to centrifuge or filter algal cells, with washing steps in fresh medium, to avoid carry-over of exudates into the test system. Furthermore, algal exudate production could be quantified over the test duration using a similar method as presented in (Koukal et al., 2009), though doing so may require increased test volumes.
In reality, there is likely a contribution to reduced toxicity over time from both the test vessels and the test organism, but quantifying the relative importance of each is not a simple task. It is also worth noting that not all toxicants produce this effect (e.g. $K_2Cr_2O_7$ as mentioned previously), suggesting that the propensity for toxicant loss relies on a detailed knowledge of chemical interaction between both the test vessel and the test organism. Finally it is worth noting that losses of toxicant to the test vessel are not ecologically relevant, compared to potential detoxification by processes unique to a particular test organism. Understanding the relative importance of each phenomenon is therefore essential, when attempting to make broader conclusions about a toxicant’s true risk to natural systems.

3.7. Conclusions

From a regulatory perspective the standard OECD 201 test appears to be sufficiently capable of determining growth inhibition for the silver nanoparticles studied in this work. However, its suitability with other nanomaterials is likely to be dependent on their physico-chemical properties (e.g. composition, size, dissolution, aggregation/agglomeration). It is evident that existing standard test guidelines are a useful way of quantifying chemical hazard, and whilst not perfect, the development of alternative tests is a laborious process which may not be able to keep pace with expanding industries such as nanotechnology. Future regulatory focus should address the ease and speed with which toxicity tests can be carried out, such as high throughput automated testing. These studies are developed with a view to increase the efficiency of testing and reducing factors such as cost, time, and space which may currently be prohibitive under the existing test requirements. Such alternatives include rapid assessments of cell density, miniaturisation, and shorter test durations (Chapter 4). Also tests which extract more data from the standard OECD test (e.g. photosynthetic yield) may be useful to inform modes of action (Chapter 5). Parallel to these developments, the field of nanotoxicology must implement standard guidelines for characterisation, which will be of great use to harmonising future research, and inform current regulation.

Furthermore, this research has highlighted a number of potential improvements to the existing OECD 201 test, which applies to both ionic and nanomaterial hazard assessment. The test protocol allows a number of alternative algal species, and two alternative media (non-prescribed medium is allowed if its use can be justified); such differences in test procedure are non-trivial and any deviation must be adequately documented and justified. The work carried out here shows how differences in media composition and pH can result in different toxicity for silver, and such results can likely be generalised to other compounds. pH is an important consideration in OECD 201 tests. The work here showed that different pH levels can induce
significant changes in both nanoparticulate and ionic toxicity. However, it was possible to retain the pH of OECD medium to < 0.1 unit of \( t_0 \) using the experimental protocol described here. Given the significant impact of pH on silver toxicity, and the ease with which pH variation can be controlled, it is recommended that future drafts of the OECD test guideline dictate more strict control of pH as a matter of test validity. Furthermore, finding ways to minimise variations between cultures should be considered an important priority to allow adequate comparisons between replicate experiments and other laboratories. From a regulatory perspective, more research is needed on the effect of algal by-products on toxicity in standard tests.

Finally, standard tests could be modified to include the effect of humic substances and pH to help build a broader picture of toxicity, with a view to increasing environmental relevancy of standard tests. A potential SOP for modifying the standard OECD 201 test to include both humic acid and pH is presented in Appendix 2. However such tests will require rigorous standardisation, and whilst of scientific interest, may be less valuable to regulatory bodies seeking to easily generate comparable results between laboratories.
Table 3.16: Silver (ionic and nanoparticles) EC50 values to a variety of algal species. EC50 values were derived from a range of standard and non-standard endpoints, after various exposure times. Endpoint, medium and derived compound are not directly comparable - see individual references for detailed methodology.

<table>
<thead>
<tr>
<th>Algal species</th>
<th>Toxicant</th>
<th>Endpoint</th>
<th>Medium</th>
<th>Timepoint (h)</th>
<th>EC50 (µg/l)</th>
<th>Derived compound</th>
<th>Author</th>
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<td><strong>Silver salts</strong></td>
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<td>Ag (100MAg-radiolabelled)</td>
<td>Growth inhibition</td>
<td>Mineral medium</td>
<td>6</td>
<td>2.80</td>
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<td>Lee et al., 2005</td>
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<td>Miao et al., 2010</td>
</tr>
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* Referred to a *Pseudokirchneriella subcapitata* or *Selenastrum capricornutum* in paper, but considered synonymous with current *Raphidocelis subcapitata* nomenclature

** estimated by Bondarenko et al., 2013
Chapter Four - Miniaturising standard algal toxicity tests

4.1. Introduction

The rapidly developing field of nanotechnology places significant responsibility on the field of toxicology/risk assessment to quantify hazard/risk at a rate which can keep pace with the development of newly synthesised nanomaterials. OECD test guidelines are pivotal within REACH, and provide a series of protocols detailing requirements of each standard test in terms of design, suitable test species and reporting. Such tests are either performed in-house by the producer/importer, or outsourced to a range of consultancy services which assess requirements under REACH. As such, the ability to satisfy the requirements of such standard tests, whilst reducing factors such as cost, time and waste (especially pertinent for nanomaterial waste), is an important aim for all stakeholders directly involved, including industry, consultancies and regulatory bodies, and the field of ecotoxicology as a whole.

Miniaturised toxicity tests have been proposed, which seek to adapt or replace standard OECD tests in order to provide alternative and possibly more efficient ways of generating toxicity data (Lammer et al., 2009; Han et al., 2015; Embry et al., 2010). In particular, the increased application of high-throughput screening (HTS) in toxicology, which seeks to develop automated methods for generating a large amount of toxicity data for the purposes of rapid risk assessment, is desirable. Such approaches have shown promise but the focus is still narrow, mostly concentrating on zebrafish tests and mammalian cell line toxicity (Nel et al., 2013). Recent work includes the use of HTS for the OECD 221 Lemna sp. growth inhibition test (OECD, 2006c; Han et al., 2015) and the OECD 202 Daphnia sp. acute immobilisation test (OECD, 2004). Some miniaturised tests have been developed into separate standard test protocols, which add additional data to determining toxicant effects on biotic systems. An example is the OECD 236 Fish embryo acute toxicity test (OECD, 2013) that was initially conceived as an alternative to OECD 203 Fish acute toxicity test (OECD 1992; Lammer et al., 2009; Embry et al., 2010). Such miniaturisation has a number of potential advantages over the standard test design. The OECD 201 test guideline recommends the use of glass flasks “of dimensions that allow a sufficient volume of culture for measurements during the test and a sufficient mass transfer of CO₂ from the atmosphere” (OECD, 2011). Test volumes of around 50-200 ml have been used in some studies (De Schamphelaere et al., 2004; Koukal et al., 2007; Navarro et al., 2008; Miao et al., 2009), but more recently smaller volumes (< 20 ml) have also been used (Aruoja et al., 2009; Wang et al., 2012; Ribeiro et al., 2014; Sekine et al., 2015; Sorensen and Baun, 2015), which have advantages in situations where toxicant supply may be limited. Miniaturised methods have additional advantages as more test substances in one
experiment, as well as include more concentrations of each test substance and/or enable greater replication can be performed, therefore increasing confidence in results. Such advantages are not limited to nanomaterials, but algal growth inhibition tests of all potential toxicants. Nevertheless it is important to note that none of these tests have yet been fully validated and so may not be acceptable for regulatory purposes.

Heldal et al. (1978), were the first to use a microplate (96 well plate) based system to test the effect of oil dispersants on *C. reinhardtii* and *Dunaliella marina* biomass after 48 hours. The term “miniaturised methods” in the broader context of the literature refers to any method using smaller test volumes/vessels than the standard OECD 201 Erlenmeyer flask test. This can include glass vials of less than 20 ml (Wang et al., 2012; Arujoa et al., 2009; Arensberg et al., 1995; Sekine et al., 2015), microplates of various sizes (Ribeiro et al., 2014; Tuominen et al., 2013; Paixão et al., 2008; Riedl and Altenburger, 2007; Pavlić et al., 2006; Eisentraeger et al., 2003; Geis et al., 2000), or petri dishes (Boudreau et al., 2003). Microplate toxicity tests have been used to assess algal growth inhibition (same endpoint as in OECD 201) but also to assess other endpoints, such as total Reactive Oxygen Species (ROS) production or enzymatic inhibition (e.g. Machado and Soares, 2012; Guan et al., 2015).

In order for miniaturised tests to be successfully adopted they should aim to not only generate comparable estimates of toxicity to traditional approaches, but also fulfil the requirements of the test. In the case of OECD 201 (OECD, 2011) these requirements are:

- A 16 fold increase in control culture biomass over the test period (equivalent to a growth rate of 0.92 d^{-1}).
- A pH change of no greater than 1.5 units in control cultures.

Previous studies have attempted to compare the data obtained from algal standard tests to that obtained using microplate/small volume methods. Arensberg et al. (1995) compared the toxicity of K_2Cr_2O_7 and 3,5-dichlorophenol to *R. subcapitata* in 20 ml glass scintillation vials and the standard OECD 201 experimental approach in 250 ml flasks, and found good concordance in toxicity data generated. In addition they found tissue culture treated microplates unsuitable for supporting *R. subcapitata* growth. The change in test medium pH was smaller in the 20 ml glass vials compared to the standard test design, although this was attributed to poor shaking of the Erlenmeyer flasks. Okamura et al. (2002) found no significant difference in *R. subcapitata* growth inhibition between 96 well microplates (unspecified material) and flasks (unspecified size) when testing a variety of environmental samples containing herbicides. Paixão et al. (2008) found a high correlation between 96 well...
Nunclon™ treated polystyrene plate and flask based growth inhibition tests for the toxicity of CuSO₄, ZnSO₄, K₂Cr₂O₇ and 3,5-dichlorophenol to \textit{R. subcapitata}. Similarly, Geis \textit{et al.} (2000) assessed the toxicity of NaCl, CuCl₂, ZnCl₂ and sodium dodecyl sulphate (NaC₁₂H₂₅SO₄) to \textit{R. subcapitata} in 24 well polystyrene microplates and 125 ml flasks, and found that the toxicity of these substances was comparable in both systems over 96 hours. Eisentraeger \textit{et al.} (2003) compared the standard OECD 201 test (100 ml, flasks) to results obtained in 24 and 96 well plates (2 and 0.1 ml, untreated polystyrene), for \textit{Desmodesmus subspicatus} across 9 test substances, after 72 hours of exposure. The mean ratio of microplate EC₅₀ value to flask EC₅₀ value was 1.09 (0.74-1.66 range) for 24 well plates, and 0.88 (0.66-1.08 range) in 96 well plates for a range of toxicants, indicating a good correlation between flasks and plate based methods. Furthermore, control algal growth rate was similar in all test systems. Although not all of these studies made attempts to specifically satisfy the requirements of the standard OECD 201 test, they demonstrate to some extent that approaches to miniaturise the assessment of algal toxicity can provide comparable results to traditional approach. However, slight variations in methodology for miniaturised assessment methods between studies (e.g. type of vessel, volumes, algae tested, incubation conditions, etc.) reveal the requirement for standardised miniaturised protocols for the adoption into the broader field of regulatory toxicity testing. It is also still unclear how dependant these differences may be on test species and test substances (see below).

The miniaturisation of standard assays may not be fully applicable to all test substances. For example Geis \textit{et al.} (2000) found the 96 hour toxicity (growth inhibition; EC₅₀) of phenol (a highly volatile aromatic compound) to \textit{R. subcapitata} was ca. 2.3 times higher when assessed in 125 ml Erlenmeyer flasks compared to 24 well plates; however the authors failed to follow OECD test guidelines exactly by using a 96 hour endpoint. Rojičková \textit{et al.} (1998) assessed the toxicity of a range of test substances (eight pharmacological drugs, three metals and a pesticide) to algae using 96 well polystyrene microplates and 100 ml flasks. The EC₅₀ value was comparable between methods, but similarities were substance specific. Substances showing the largest difference in sensitivity between tests (> 2x higher in microplates) included a disinfectant (Virkon S) and an antibiotic (Egocine).

Despite their potential advantages, miniaturised OECD 201 methods have not been broadly applied for assessment of nanomaterial toxicity to algae, with the exception of a few studies (e.g. Hunde-Rinke and Simon, 2006; Aruoja \textit{et al.}, 2009; Lee and An, 2013). The purpose of this chapter is to perform comparisons between nanomaterial toxicity to \textit{R. subcapitata} using data generated in the standard OECD 201 flasks and 24 well plates. A
number of practical considerations were necessary in the development of such methods, and these are described below.

1) Evaporation

Testing using microplates requires smaller volumes; for example testing using a 24 well plate typically uses a volume of 2 ml, compared to 20-100 ml in flasks (depending on flask volume). With such small volumes comes an increased susceptibility to evaporation. Pilot studies carried out in this project using both 24 and 96 well plates resulted in complete evaporation of the test medium if plates were not sealed, an effect that has been noted by others (Blaise et al., 1998). Partial evaporation may also artificially increase cell density (Arensberg et al., 1995), so measures must be taken to limit evaporation as much as possible. A number of approaches have been proposed to minimise evaporation. Early studies placed plates inside heat-sealed plastic bags (Blaise et al., 1982), however reading samples would have required opening and resealing bags each time if multiple timepoints were tested. Increasing relative humidity of the entire incubator is another method to reduce evaporation (Arensberg et al., 1995), however such methods are not well described nor tested. A more modern and simple approach is to use gas permeable film (e.g. Parafilm) which retains humidity within the plate itself but allows CO₂ and O₂ to diffuse across to allow growth (Eisentraeger et al., 2003; Riedl and Altenburger, 2007). The use of such films may limit gas exchange and therefore growth, as Van Wagenen et al. (2014) observed an increase in algal growth rate observed upon the puncturing of such film, used to cover algal cultures. Such effects, however, may also be attributed to increased evaporation as suggested above (i.e. a reduced medium volume and concentration of cell density). Furthermore, evaporation may change the ionic strength of the test solutions and so cause artefacts in interpreting growth inhibition with compounds sensitive to ionic strength. Additional steps to minimise evaporation include filling unused wells with distilled water (Lukavský et al. 1992) and using plate lids designed to seal each well individually (Eisentraeger et al., 2003).

2) Gas exchange

In the context of algal toxicity tests gas exchange is important for two reasons: to supply adequate CO₂ as a substrate for algal photosynthesis, and to buffer growth induced changes in pH. Sealing plates to prevent evaporation may compete with needs for adequate gas exchange; ideally film used to seal plates should be adequately permeable to allow unrestricted gas exchange and pH buffering (see Section 3.1.3. for a discussion of pH buffering in algal toxicity tests), but not so permeable as to allow moisture loss due to evaporation. Thellen et al. (1989) suggested that improved gas exchange (in the form of enriching the test
chamber with CO$_2$) in 96 well microplates enhanced microalgal growth, but had no effect on toxicity estimates (growth inhibition; EC$_{50}$) for CdCl$_2$ and phenol, compared to testing using the traditional flask test design. However these authors did not measure pH in the microplates and only tested two chemical substances, so it is not possible to conclude from this study alone that gas exchange is not an essential concern. Arensberg et al. (1995), conducted tests in 20 ml glass vials, and suggested shortening test duration from 72 hours to 48 hours to prevent large changes in pH. However the miniaturised tests performed exhibited the acceptable control pH range (< 1.5 units) and growth rate (1.72-2.37 d$^{-1}$) over a three day period. This again, was suggested with reference to the similar 48 and 72 hour EC$_{50}$ values generated for the test substances studied (K$_2$Cr$_2$O$_7$ and 3,5-dichlorophenol). However, the potency of substances can change over time. For example both silver nanomaterials and ionic silver, typically exhibit declining (with time) toxicity over a standard 72 hour OECD test (Sekine et al. 2015; Section 2.5.6). Consistent use of the 72 hour timepoint in miniaturised tests will also facilitate making comparisons with existing studies which have assessed toxicity according to the OECD 201 protocol. Thus, it is not advisable to shorten the test duration when developing miniaturised algal tests.

3) Adherence of the test substance to test vessel

In all test systems, general concerns have been raised regarding sorption of toxicants due to the different test container material (i.e. plastic vs glass) and increased surface area to volume ratio associated with the use of smaller vessels (Blaise et al., 1992; Sekine et al., 2015). With particular reference to both ionic silver and silver nanomaterials, some studies have justified the use of polycarbonate test vessels (plates and flasks) to reduce sorption and consequently losses of dissolved silver to the vessel surface (Fortin and Campbell, 2001; Lee et al., 2004; 2005; Hiriart-Baer et al., 2006; Navarro et al., 2008; Chen et al., 2013). Conversely, many other 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), this effect is magnified in glass flasks with a larger surface area to volume ratio. Furthermore, silver nanomaterial adsorption is highly dependent on physicochemical properties of the nanomaterials themselves, and composition and size of the test vessel. For example Sekine et al., 2015 found that positively charged silver nanoparticles (branched-polyethyleneimine coated; 10 and 60 nm; 50 and 180 µg/l) were almost completely removed from solution (> 90% removal for all nanoparticles, with the exception of 180 µg/l 10 nm particles, which exhibited 45-70% loss) after 72 hours when contained in 30 ml polycarbonate vials. Conversely, negatively charged (tannic acid;
same sizes/concentrations) remained almost fully suspended (<25% loss, regardless of size/concentration) over the same time period in the same vessels. When combined with results for Ag⁺, the choice of test vessel material is non-trivial, but highly complicated by an extensive range of potentially interacting parameters (e.g. particle vs ion behaviour, concentration, particle size, particle charge, container geometry, container material, etc.).

Given such behaviour, and its potential to modify toxicity estimates, neither glass nor plastic can be said to be more or less suitable for determining toxicity, since it also depends on the chemical being tested. What is apparent is the need for studies to explicitly report test container characteristics (type, coating, geometry and volume) as well as incubation regimes, in order to make fully informed comparisons between toxicity data obtained from different laboratories and approaches. Importantly, despite these concerns, miniaturised methods have shown to be remarkably comparable with the standard OECD 201 method for a number of test substances (Eisentraeger et al., 2002; Pavlić et al., 2006).

4) Volatile compounds

Issues with testing the toxicity of volatile compounds in miniaturised test systems have been identified in a number of studies, with the most common being diffusion of volatile toxicants into adjacent unsealed test vessels (Thellen et al., 1998; Eisentraeger et al 2003; Riedl and Altenburger, 2007). As mentioned previously, Geis et al. (2000) found that 24 well polystyrene plates overestimated phenol toxicity relative to 125 ml Erlenmeyer flasks, indicating lower sensitivity of well plates; an observation authors hypothesised to be due to a higher surface area to volume ratio in well plates, which may have caused phenol to volatise out of solution more rapidly.

Whilst silver and other nanomaterials are non-volatile, such concerns may be of relevance to the broader adoption of miniaturised methods for a wider variety of test substances. However, it should be noted that the OECD 201 growth inhibition test is not in any case designed to assess toxicity of volatile compounds, for which there are a number of proposed modifications (ISO, 1998; 2006; OECD, 2000). For the purposes of this chapter, which aims to compare the suitability of miniaturised methods as a potential alternative to the OECD 201 standard test with specific reference to silver nanoparticles, no further consideration will be given to applicability of either test to volatile substances.
4.1.1. The development of rapid methods of determining cell density

In addition to practical considerations for the optimisation of microplate tests, broader consideration has been given to implementing rapid and accurate surrogates for assessment of cell growth which may be of benefit to both flask and microplate based tests.

OECD test 201 assesses algal growth over a 72 hour period (OECD, 2011). Whilst the test states cell density as the primary method of assessing growth, it acknowledges the methodological difficulty of such methods and suggests the use of other parameters. Cell counts may be performed manually using a microscope and haemocytometer, or other methods such as electronic particle counters. However these techniques also have disadvantages; manual counting is highly variable and subject to inter-observer error, and particle counters are costly and require high sample volumes to account for the high error associated with low algal densities (Mayer et al., 1997). In addition, cell counts (both manual and electronic) may be confounded in the presence of highly hetero-aggregating/agglomerating nanomaterials which may include algal cells, making enumeration difficult (Hartmann et al., 2012). The use of surrogate methods that can effectively quantify algal growth, without being subject to the limitations of direct counting, are also listed in OECD 201 as suitable methods to assess algal cell density. Such methods must not only show high correlation with cell counts, but also be of sufficient sensitivity to detect low densities of algae required in standard growth inhibition tests (Nyholm and Petersen, 1993; Fai et al., 2007).

Three methods have been used in the literature and these are described below.

1) Optical density/absorbance

Assessment of algal growth can be achieved via measurement of optical density using a spectrophotometer. This photometric technique detects changes in cell density based on the light scattering and absorbance of an algal culture at a given wavelength, usually the one of highest absorbance (Griffiths et al., 2011). In algal studies, this wavelength usually corresponds to the two absorption maxima of Chl \(a\), typically 400-460nm and 650-680nm (Takagi et al., 2000; An et al., 2003; Bopp and Lettieri, 2007; Chiu et al., 2007). An increase in absorbance should therefore be indicative of an increase in algal cell density. Absorbance values can be correlated with cell counts and a standard curve derived, which can be used to calculate cell density. Such methods typically lack the sensitivity to quantify low cell densities required for time zero \(t_0\) measurements of the inoculum of standard growth inhibition tests, but this can be overcome by increasing the path length of the cuvette (both 1 and 10 cm cuvettes are available). In addition, cultures with an absorbance of < 1 require dilution to prevent underestimation of density (Eisentraeger et al., 2003; Kviderová, 2009; Van Wagenen et al.,
Cell density can also be underestimated if cultures undergo a change in pigment/cell size or shape over their growth period (Clesceri et al., 1998; Griffiths et al., 2011), or if turbid environmental samples or samples containing nanomaterials are used (Eisentraeger et al., 2003).

2) In vitro Chl$_a$ fluorescence

Chlorophyll a (Chl$_a$) is a ubiquitous photopigment found in all phytoplankton. By extracting Chl$_a$ from algal cells, followed by its quantification using fluorometry (or other similar methods), the photosynthetic pigment concentration can be used as a surrogate to cell density (Mayer et al., 1997). An adapted method of Mayer et al. (1997) was originally used as the principle method of cell density assessment in algal studies at Heriot Watt University (Kinross, personal communication), and in Chapter 3. This approach has a high precision at low cell densities, but requires pigment to be extracted in the absence of light for hours-days (depending on extraction solvent). A whole water extraction technique, originally described by Mayer et al. (1997), offered a significant improvement over previous methods, which required time consuming filtration/centrifugation steps (Shoaf and Lium, 1976). This method has since been adapted for use in other algal toxicity tests (Halling-Sorensen, 2000; Mulderij et al., 2003; Arujoa et al., 2004; Christensen et al., 2006), including nanomaterials (Hartmann et al., 2010; Van Hoecke et al., 2013; Sekine et al., 2015; Sorensen and Baun, 2015, Kalman et al., 2015).

3) In vivo Chl$_a$ fluorescence

In vivo Chl$_a$ relies on the interaction of light with the photosynthetic apparatus in the chloroplasts in the algal cells, in order to quantify density. Light energy of a given wavelength is absorbed by light harvesting complexes (a group of proteins, including chlorophyll $a$) and transferred to the photosystem reaction centres (PSII and PSI). Through the oxidation of water, PSII and PSI excite the free electrons and create a proton gradient, and so the reducing energy for CO$_2$ fixation (Rohacek and Bartak, 1999). The measured fluorescence is the result of remission of light at a higher wavelength, as the excited electron returns to its original state in the chlorophyll $a$ molecule (Falkowski and Raven, 2007). This method has been used to non-destructively estimate algal cell density in many laboratory studies (Eisentraeger et al., 2003; Huot and Babin, 2011; Van Wagenen et al., 2014; Skjelbred et al., 2015). Fluorescent cell density determinations can be obtained even in the presence of interference from medium components (humic substances, sewage samples, etc.), provided their signal remains constant (Eisentraeger et al., 2003). Whilst changes in the ratio of chlorophyll $a$ concentration to in vivo fluorescence can occur, growth rate may be calculated without the need for a conversion factor (Tunzi and Porcella, 1974).
The use of any of these three approaches provides alternatives to the quantification of algal cell density, by saving time and reducing variability. In the case of optical density and \textit{in vivo} Chl\textsubscript{a}, these methods show potential for microplate methods (Geis \textit{et al}., 2000; Eisentraeger \textit{et al}., 2003; Ribiero \textit{et al}., 2014; Podevin \textit{et al}., 2015). Eisentraeger \textit{et al}.
(2003) and Van Wagen \textit{et al}.
(2014) assessed algal growth via measurements of optical density and \textit{in vivo} Chl\textsubscript{a} in microplates and found that absorbance based approaches lack the sensitivity of measurement of \textit{in vivo} Chl\textsubscript{a} fluorescence. Thus, they suggested that measurement of optical density is unable to effectively estimate the early growth phase of cultures in microplates, as path length cannot be modified sufficiently to account for low algal cell density. Whilst some authors have measured \textit{in vivo} Chl\textsubscript{a} to successfully determine the toxicity of nanomaterials to algae in both flask and microplate based tests (Hunde-Rinke and Simon, 2006; Lee and An, 2013), other studies still rely on optical density (Ribeiro \textit{et al}., 2015; Golubev \textit{et al}., 2016). Through combining both toxicity test systems (i.e. flasks or microplates), and methods of measuring cell density, a variety of approaches can be used to determine concentration response data. As such, comparing results between such tests is problematic due to a lack of standardisation.

Fast, accurate and non-invasive methods of enumerating cell density can be considered an essential part of the development and widespread adoption of miniaturised algal growth inhibition tests. Furthermore, validation of such methods would enable the use of automated testing units that can perform high throughput screening of not just toxicity (Nel \textit{et al}., 2013; Eisentraeger \textit{et al}., 2003), but also in supporting optimisation of growth parameters for algae of biotechnological value (Van Wagenen \textit{et al}., 2014).

\textbf{4.2. Aims and objectives}

The aims of this chapter were as follows:

- To assess the suitability of using three different surrogate methods for rapidly determining \textit{R. subcapitata} cell density. To achieve this cells were cultured in the standard test system (≈50 ml of culture in 250 ml Erlenmeyer flasks) to obtain a growth curve, and algal density over time were determined using cell counts, optical density, \textit{in vitro} and \textit{in vivo} Chl\textsubscript{a}. Assessment of algal growth using optical/fluorescent measurements were plotted against manual cell count data in order to identify suitability of such methods for determining algal growth.
- To compare the sensitivity of the standard OECD 201 growth inhibition test conducted in flasks to that of 24 well microplates. To achieve this concentration response data were simultaneously assessed in both flasks and microplates for 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), AgNO$_3$ and NM300K. In addition, the use of two humic acid concentrations and two pH levels, at a fixed toxicant concentration, provided more data on the comparative sensitivity of both test designs.

As the focus of this chapter is the assessment of the suitability of applying miniaturisation methods to OECD 201 algal growth inhibition test, comparisons will be made between tests in OECD medium only.

4.3. Hypotheses

4.3.1. Research Hypotheses

- Surrogate measurements of cell density (optical density, $\textit{in vitro}$ Chl$_a$ and $\textit{in vivo}$ Chl$_a$) show a high correlation with cell counts.

- In toxicity studies (using DCMU, AgNO$_3$, NM300K), 250 ml flask based $\textit{in vivo}$ Chl$_a$ 72 hour EC$_{50}$ values, and control growth rate measurements are not different to those measurements made with flask based $\textit{in vitro}$ Chl$_a$, and to $\textit{in vivo}$ Chl$_a$ measurements in 24 well plates.

- Flasks and microplates show a similar relationship when silver toxicity (AgNO$_3$ and NM300K) is assessed in medium containing Suwannee River humic acid (5 and 50 mg/l SRHA), and medium buffered to pH 6 and 8 (i.e. compared to results in Chapter 3).

4.3.2. Null Hypotheses

- Surrogate measurements of cell density show no correlation with cell counts.

- In toxicity studies, EC$_{50}$ and control growth rate measurements are not comparable between measurements made in flasks using $\textit{in vitro}$ Chl$_a$, flasks using $\textit{in vivo}$ Chl$_a$, and in 24 well plates using $\textit{in vivo}$ Chl$_a$.

- Flasks and microplates show a no similar relationship when silver toxicity (AgNO$_3$ and NM300K) is assessed in medium containing Suwannee River humic
acid (5 and 50 mg/l), and medium buffered to pH 6 and 8 (i.e. compared to results in Chapter 3).

4.4. Methods

4.4.1. Comparisons of surrogate methods used to estimate cell density

Triplicate cultures of *R. subcapitata* were inoculated in OECD medium (cell density 5 x 10⁴ cells/ml) in 250 ml borosilicate flasks and incubated under conditions described in Section 3.4.2. Samples were taken for the four measures of cell density (see below): cell counts, optical density, *in vitro* Chlₐ and *in vivo* Chlₐ. Samples were taken at 0, 6 and 24, 30, 48, 54, 72, 78, 96 and 102 hours, when the stationary phase of growth was reached.

4.4.1.1. Cell counts

Samples were taken for cell counting by pipetting 1 ml of the algal culture into a microfuge tube containing 0.05ml of formaldehyde. Cells were counted under a light microscope (400x magnification, Leitz Weitzlar) using a haemocytometer.

4.4.1.2. Optical density

A volume of 25 ml of algal culture was measured in a 10 cm path length glass cuvette (Hellma Analytics). Optical density was measured at 685 nm and 650 nm using a dual beam UV/visible spectrophotometer (Jenway, model 6715). The equipment was blanked using cell free OECD medium prior to taking the measurement. Samples were then returned to the parent flask to conserve volume.

4.4.1.3. *In vitro* Chlₐ

A simplified, whole-water Chlₐ extraction was performed based on methods adapted from *Mayer et al.* (1997) (see Section 3.4.3. for detailed methodology).

4.4.1.4. *In vivo* Chlₐ

A triplicate volume (200 µl) of each algal culture was pipetted into the wells of a black 96 well plate. Fluorescence was read at Ex: 435 nm and Em: 690 nm using a SpectraMax M2 Fluorescence Plate Reader (Molecular Devices, USA). The plate reader was set to shake samples for 5 seconds prior to the reading.
4.4.2. Comparisons of toxicity data between flasks and 24 well plates

Algae/toxicant suspensions were prepared in 160 ml volumes in glass flasks following the methods described in Section 3.4.3. From each of these stock suspensions, 1.5 ml was aliquoted into the wells of an untreated transparent, flat bottomed polypropylene 24 well plate (Corning® Costar®, USA) in triplicate (or six for controls). Test concentrations were aliquoted into 24 well plates using the same layout as described in Eisentraeger et al. (2003). For concentration response studies, three test substances were studied: AgNO₃ (0.2-20 µg/l Ag), NM300K (30-150 µg/l Ag), and DCMU (0.2-100 µg/l). DCMU was used as a reference toxicant for photosynthetic inhibition studies (Chapter 5), so was only tested in concentration response experiments. NIST-Traceable certified reference material DCMU was supplied in methanol (SPEX CertiPrep; 1000 µg/ml), and master stocks were made to a concentration of 1000 µg/l DCMU (v/v), by pipetting 50 µl of the DCMU master stock into 50 ml 18 mΩ water. Methanol only control stocks were prepared by pipetting 50 µl of MeOH into 50 ml 18 mΩ water. DCMU master stocks and methanol controls were sonicated and serially diluted as described in Section 2.4.1.

Growth in flasks was measured every 24 hours using both in vitro and in vivo Chlₐ methods (Section 4.3.1c and d). Growth in microplates was measured every 24 hours using an adapted in vivo Chlₐ method described in Section 4.3.1d so it was suitable for use in a 24 well plate. Fluorescence was read in situ without transferring into a black well plate. To test for lateral fluorescence interference between adjacent wells of the clear well plates, cultures in transparent plates were transferred to black walled, transparent bottomed 24 well plates (VisiPlate, PerkinElmer, UK) and re-read using the same settings (at 24, 48 and 72 hours). Plates were closed with a transparent polypropylene cover and sealed with Parafilm between measurements.

In humic acid and pH studies, a single toxicant (either NM300K or AgNO₃) was investigated across different medium conditions (i.e. 0, 5 and 50 mg/l SRHA or unbuffered, pH 6 and pH 8 medium) (see Section 3.3.4. for detailed methodology for flasks). As with concentration response studies, 1.5 ml per well was aliquoted from each flask in triplicate (or six for controls) into transparent, flat bottomed polypropylene 24 well plate (Corning® Costar®, USA). Test concentrations were added into 24 well plates using a modified method to that described in Eisentraeger et al. (2003) (Table 4.2 and Table 4.3). Growth in either flasks or wells were measured every 24 hours using the same methods described for concentration response studies (i.e. flasks in vitro Chlₐ and in vivo Chlₐ; 24 well plate in vivo Chlₐ).
Table 4.1: 24 well plate set up for humic acid/silver growth inhibition studies with *R. subcapitata* in OECD medium. Toxicant concentrations comprised of either NM300K [190 µg/l Ag] or AgNO₃ [6 µg/l Ag]. Two humic acid concentrations of 5 and 50 mg/l Suwannee River humic acid (SRHA) were used to investigate the effect on toxicity. All test volumes = 2 ml per well. Cell free blanks of OECD medium containing 0, 5, and 50 mg/l SRHA were placed in wells A4, A5, and A6 respectively to measure background fluorescence.

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<tr>
<td>A</td>
<td>Toxicant free/humic acid free control</td>
<td>Blank 0mg/l SRHA</td>
<td>Blank 5mg/l SRHA</td>
<td>Blank 50mg/l SRHA</td>
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<tr>
<td>B</td>
<td>Toxicant only</td>
<td>50 mg/l SRHA control (toxicant free)</td>
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<tr>
<td>C</td>
<td>5mg/l SRHA control (toxicant free)</td>
<td>Toxicant + 50 mg/l humic acid</td>
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<td>D</td>
<td>Toxicant + 5 mg/l humic acid</td>
<td>Toxicant free/humic acid free control</td>
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Table 4.2: 24 well plate set up for pH/silver growth inhibition studies with *R. subcapitata* in OECD medium. Toxicant concentrations comprised of either NM300K [190 µg/l Ag] or AgNO₃ [6 µg/l Ag]. Two pH levels of 6 and 8 were used (buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to investigate the effect on toxicity. All test volumes = 2 ml per well. Cell free blanks of unbuffered OECD medium, medium buffered to pH 6, and medium buffered to pH 8 were placed in wells A4, A5, and A6 respectively, to measure background fluorescence.

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<td>A</td>
<td>Toxicant free/unbuffered control</td>
<td>Blank unbuffered</td>
<td>Blank pH 6</td>
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<td>B</td>
<td>Toxicant only</td>
<td>pH 8 control (toxicant free)</td>
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<td>Toxicant at pH 8</td>
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<td>D</td>
<td>Toxicant at pH 6</td>
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4.4.3. Statistical analysis

Details of statistical testing software and procedures can be found in Section 2.4.5. In order to compare methods of estimating algal cell density in 102 hour growth curves, the relationship between the three surrogate methods of estimating density were tested for their ability to predict cell count using a standard linear regression.

In 102 hour growth curve studies, *R. subcapitata* maximum growth rate was calculated, for each method of estimating density, between the two timepoints which showed the largest increase in cell density (indicating exponential growth phase; 30-72 hours), using the same equation as in Section 3.4.3:

$$\mu_{30-72} = \frac{(\ln X_{72} - \ln X_{30})}{t_{72} - t_{30}}$$

Where: $\mu_{30-72}$ = specific growth rate from 30 hours to 72 hours; $X_{30}$ = cell density (or surrogate parameter) at 30 hours; $X_{72}$ = cell density (or surrogate parameter) at 72 hours.

Specific growth rate was converted to daily specific growth rate by dividing by 24 (to facilitate comparison with other chapters), and this was then compared statistically between density estimation methods.

In concentration response and humic/pH studies, *R. subcapitata* control growth rate was compared at each timepoint, across the three test methods (flasks *in vitro* Chl$_a$, flasks *in vivo* Chl$_a$, 24 well plate *in vivo* Chl$_a$). Comparisons between flasks *in vitro* Chl$_a$, flasks *in vivo* Chl$_a$, 24 well plate *in vivo* Chl$_a$ growth rate were plotted with a factor of 5 difference boundary marked as in Eisentraeger *et al.* (2003). For concentration response data a probit model was fitted to growth inhibition data generated at each timepoint. EC$_{50}$ (μg/l) was compared between both media and timepoint, for DCMU, NM300K, AgNO$_3$.

*R. subcapitata* growth rate was compared between test designs, in humic acid and pH studies (i.e. all control treatments). Any control cultures showing no growth (i.e. no positive change in cell density over time) were removed from growth rate and dependent concentration response analyses (i.e. a total of one humic acid/NM300K study in a 24 well plate, and a single unbuffered replicate in one 24 well plate pH/NM300K study). Comparisons between flasks *in vitro* Chl$_a$, flasks *in vivo* Chl$_a$, 24 well plate *in vivo* Chl$_a$ growth inhibition across all tests (i.e. NM300K and AgNO$_3$; concentration response, humic acid and pH studies) were plotted with a factor of 5 difference boundary marked as in Eisentraeger *et al.* (2003).
4.5. Results

4.5.1. Comparisons of surrogate methods of estimating cell density

All four methods of determining cell density showed the same general pattern of growth in *R. subcapitata* (Figure 4.1). Lag phase lasted around 30 hours, followed by an exponential growth period of around 40 hours after which the stationary phase was observed from 72 hours onwards. Assessment of cell density via measurement of *in vitro* Chlₐ (Figure 4.1c) showed a rapid increase into exponential growth between 30 and 48 hours, compared to the other three methods which showed a more gradual increase before the onset of full exponential growth from 48 hours onwards (Figure 4.1a, b and d). Assessment of cell density via measurement of optical density (Figure 4.1b) showed no cessation of exponential growth at 72 hours. Cell count data (Figure 4.1a) showed a slight reduction in growth after 72 hours. *In vitro* and *in vivo* Chlₐ (Figure 4.1c and d) showed a marked cessation of growth after 72 hours.

![Graphs showing cell count, optical density, and Chlₐ measurements over time](image)

**Figure 4.1:** Change *R. subcapitata* cell density (starting density 5 x 10⁴ cells/ml) in cultures grown in OECD medium, as determined by a) manual cell counts, b) optical density at 685 nm, c) *in vitro* Chlₐ, d) *in vivo* Chlₐ (Ex/Em: 435/685 nm). Data expressed as mean, and standard error of the mean (n = 3)
Maximum growth rate (i.e. change in cell density between 30 and 72 hours) was significantly different between methods of estimating cell density (F = 209.85, p < 0.001). Maximum growth rate was significantly higher when estimated using cell count data (1.70 ±0.01 d⁻¹) than all other methods. Growth rate derived from optical density (1.40 ±0.004 d⁻¹) was significantly higher than in vitro (1.28 ±0.03 d⁻¹) and in vivo (1.34 ±0.02 d⁻¹) Chlₐ, which were not significantly different from each other (Figure 4.2).

![Figure 4.2: Maximum growth rate (d⁻¹) of *R. subcapitata* in OECD medium, measured by cell count, optical density (absorbance at 685 nm), in vitro Chlₐ (µg/l) and in vivo Chlₐ (Ex/Em: 435/685 nm) in flasks. Data expressed as mean growth rate and standard error of the mean (n = 3). Letters denote significant differences between each method of estimating cell density (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.](image)

All three surrogate methods of estimating algal density displayed a strong positive linear relationship with cell count (p < 0.001) (Figure 4.3). Optical density and in vivo Chlₐ (Figure 4.3a and c) showed the best predictive power of cell density (r² = 0.96 and r² = 0.95 respectively), whereas in vitro Chlₐ showed a slightly weaker predictive power (r² = 0.83). There was a data gap for cell counts between 2 x 10⁶ and 5 x 10⁶ cells/ml, due to rapid exponential growth between 30 and 48 hours.
4.5.2. Control performance in flasks and well plates

*R. subcapitata* growth rate was significantly different between flasks and microplates at 24 hours (*F* = 38.25, *p* < 0.001), 48 hours (*F* = 71.50, *p* < 0.001), and 72 hours (*F* = 129.56, *p* < 0.001). *In vivo* Chl*α* determined algal growth rate in 24 well plates (1.19 ± 0.03 d⁻¹) was significantly lower than in flasks (measured using either *in vivo* or *in vitro* Chl*α*) (Figure 4.4). In the flask based experimental design, growth rate estimated using *in vivo* Chl*α* (1.49 ± 0.02 d⁻¹) was significantly lower than that of *in vitro* Chl*α* estimates. Whilst both *in vivo* Chl*α* methods of estimating cell density (in 24 well plates and in flasks) did not differ significantly over time, estimates of growth rate in flasks using *in vitro* Chl*α* showed a small but significant decrease in growth rate by 72 hours (1.55 ± 0.02 d⁻¹), compared to 24 and 48 hour estimates (1.86 ± 0.04 d⁻¹) (Figure 4.4). All methods fulfilled the minimum growth requirement of OECD 201 test guidelines (> 0.92 d⁻¹) with 72 hour growth rates of 1.2 (±0.01), 1.55 (±0.02), and 1.46 (±0.01) d⁻¹ for 24 well plates *in vivo* Chl*α*, flask *in vitro* Chl*α* and flask *in vivo* Chl*α*, respectively. Cell counts were not performed since they were considered not required given the high correlation of the different surrogate methods with manual cell counts (Figure 4.3), and in the case of 24 well plates, insufficient volumes were available to perform all approaches.
Figure 4.4: Experimental growth rate of *R. subcapitata* in control cultures of OECD medium over 72 hours, estimated using different test systems; at 24 well plate *in vivo* Chl\(_a\) (black bars), flasks *in vitro* Chl\(_a\) (grey bars) and Flasks *in vivo* Chl\(_a\) (white bars). Dashed line represents minimum required 72 hour growth rate, according to OECD test guideline (0.92 d\(^{-1}\)). Data expressed as mean growth rate and standard error of the mean (n = 24). Letters denote significant differences between methods at each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

*R. subcapitata* control growth rates, across all experiments in 24 well plates, were compared with those measured in flasks by *in vitro* (Figure 4.5) and *in vivo* (Figure 4.6) methods. Growth rates across all methods were more variable at the beginning of the test (i.e. at 24 hours) than at the end (i.e. at 72 hours), as shown by the distribution of growth rates which become more tightly clustered over time (Figures 4.5 and 4.6). Growth rates were lower in well plates, but did not deviate from flasks by more than a factor of 5 (within dotted line boundary – Figure 4.5) except in the case of three 24 hour growth measurements. Lower variation in growth rate was observed in 24 well plates compared to both flask based measurements (Figures 4.5 and 4.6). These data indicate that all three methods are suitable for determining growth rate in *R. subcapitata*, especially at later timepoints (i.e. 72 hours), used in the standard OECD 201 test.
Figure 4.5: Comparison between *R. subcapitata* control growth rate (μ) in OECD medium between the 24 well plate test and the OECD standard method (conducted in flasks, *in vivo* Chl*α* extraction) after 24 hours (black circles), 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean toxicant free control growth rate of a single experimental condition (six replicates) and standard error of the mean; n = 39. Solid line represents equal growth rate boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results. Dashed lines represent minimum required 72 hour growth rate, according to OECD test guidelines (0.92 d⁻¹).
Figure 4.6: Comparison between *R. subcapitata* control growth rate ($\mu$) in OECD medium between the 24 well plate test and the OECD standard method (conducted in flasks, *in vitro* Chl$\alpha$ extraction) after 24 hours (black circles), 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean toxicant free control growth rate of a single experimental condition (six replicates) and standard error of the mean; $n = 39$. Solid line represents equal growth rate boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results. Dashed lines represent minimum required 72 hour growth rate, according to OECD test guidelines (0.92 d$^{-1}$).

*R. subcapitata* growth rate was also compared within flasks, based on the two methods of enumeration (Figure 4.7). As expected, growth rates were more tightly correlated. *In vitro* Chl$\alpha$ extraction was more variable, especially at earlier timepoints, than *in vivo* methods.
Figure 4.7: Comparison between *R. subcapitata* control growth rate (µ) in OECD medium between from two methods of measuring growth in the same OECD standard test system (flasks; *in vivo* and *in vitro* Chlₐ) after 24 hours (black circles), 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean toxicant free control growth rate of a single experimental condition (six replicates) and standard error of the mean; n = 42. Solid line represents equal growth rate boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results. Dashed lines represent minimum required 72 hour growth rate, according to OECD test guidelines (0.92 d⁻¹).

4.5.3. Toxicity estimates

The impact of DCMU (Figure 4.8, Table 4.3), NM300K (Figure 4.9, Table 4.4) and AgNO₃ (Figure 4.10, Table 4.5) was assessed in flasks and 24 well plates. For all test substances, a concentration (Figure 4.8-4.10) and time (Table 4.3-4.5) dependent inhibition in algal growth was observed. Methanol only (used to disperse DCMU) control studies yielded no toxicity at the highest equivalent concentration as in DCMU studies (Data not shown). In general, assessment of growth inhibition (EC₅₀) at 72 hours using *in vitro* Chlₐ as a measure of algal growth was higher than that observed for the two *in vivo* methods used to assess algal growth in flasks and microplates. When comparing between test systems using the same measurement (*in vivo* Chlₐ) concentration response data were more closely coupled.

DCMU toxicity was more stable over the test duration (Table 4.3) compared to either forms of silver (Tables 4.4 and 4.5); that is the EC₅₀ value did not increase as sharply over time for DCMU, indicating a similar toxicity across the timepoints studied. When determining DCMU toxicity using *in vitro* Chlₐ in flasks, a small but significant decrease in the EC₅₀ value was
observed ($F = 288.44, p < 0.001$), between 24 ($63.76 \pm 2.01 \, \mu g/l$) and 72 hours ($43.20 \pm 1.36 \, \mu g/l$) (Table 4.3). However when flask based toxicity measured using \textit{in vivo} Chl$_a$, no similar significant increase in the EC$_{50}$ value was observed. Conversely, when using \textit{in vivo} Chl$_a$ methods in the well plate test, the EC$_{50}$ value significantly increased over time ($F = 9.79, p = 0.013$), with a 24 and 72 hour EC$_{50}$ value of 17.44 ($\pm 0.68$) and 24.02 ($\pm 0.79$) $\mu g/l$ respectively.

At 72 hours (i.e. the standard OECD 201 test timepoint), \textit{in vivo} Chl$_a$ derived DCMU EC$_{50}$ values (Flasks $27.08 \pm 0.82 \, \mu g/l$; Well plates $24.02 \pm 1.32 \, \mu g/l$) were significantly lower than that obtained from flask based \textit{in vitro} Chl$_a$ extraction ($43.20 \pm 0.60 \, \mu g/l$) ($F = 126.33; p < 0.001$) (Figure 4.8). These results indicate that when using \textit{in vivo} Chl$_a$ as a surrogate method to estimate cell density in DCMU toxicity tests, both 24 well plate and flask based test designs show a comparative level of sensitivity. Furthermore, \textit{in vivo} Chl$_a$ sensitivity is greater for both test systems than flask based measurement of \textit{in vitro} Chl$_a$. When using \textit{in vitro} Chl$_a$ as a measurement of algal growth, DCMU toxicity appears to slightly decrease over 72 hours, whereas for \textit{in vivo} Chl$_a$ it either slightly increases (as in the case of 24 well plates) or stays the same (in the case of flasks).
Figure 4.8: Impact of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) [0.2-100 µg/l] on *R. subcapitata* growth rate inhibition at 72 hours in OECD medium, in flasks/in vivo Chlₐ (black circles), flasks/in vitro Chlₐ (grey squares) and 24 well plates/in vivo Chlₐ (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Table 4.3: Probit modelled effective concentrations of DCMU [0.2-100 µg/l] on *R. subcapitata* growth rate (24, 48, and 72 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model DCMU induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 1, one experiment with three replicates). Superscript letters denote significant differences in the EC₅₀ value within test system, between each timepoint (One way ANOVA; p < 0.05); EC₅₀ values not sharing the same superscript letter were significantly different. No significant change in EC₅₀ value was observed over the test duration for flasks in vivo.

<table>
<thead>
<tr>
<th>Method</th>
<th>24 hour</th>
<th>48 hour</th>
<th>72 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flasks in vitro</td>
<td>63.76 (±2.01)ᵃ</td>
<td>47.42 (±1.49)ᵇ</td>
<td>43.20 (±1.36)ᶜ</td>
</tr>
<tr>
<td>Flasks in vivo</td>
<td>31.54 (±1.09)</td>
<td>25.17 (±0.79)</td>
<td>27.08 (±0.82)</td>
</tr>
<tr>
<td>24 well plates in vivo</td>
<td>17.44 (±0.68)ᵇ</td>
<td>18.57 (±0.73)ᵇ</td>
<td>24.02 (±0.79)ᵃ</td>
</tr>
</tbody>
</table>
NM300K caused a concentration dependent increase in growth inhibition at 72 hours in both test systems (flasks and microplates). Furthermore, the EC\textsubscript{50} values for NM300K increased over the test duration, in both test systems (flasks and microplates), when \textit{in vitro} and \textit{in vivo} Chl\textsubscript{a} was used to determine cell density (Table 4.4). However, whilst EC\textsubscript{50} values increased over time in all test conditions, only flask based methods showed this effect to be significant compared to the other two methods (\textit{in vitro} Chl\textsubscript{a} F = 3.83, p = 0.045; \textit{in vivo} Chl\textsubscript{a} F = 3.81, p = 0.046); with a 24 hour EC\textsubscript{50} value of 44.87 (±1.57) and 30.79 (±1.84) µg/l, and a 72 hour EC\textsubscript{50} of 117.21 (±1.80) and 94.86 (±1.64) µg/l, for \textit{in vitro} and \textit{in vivo} Chl\textsubscript{a} methods respectively. Unlike DCMU, no significant difference in 72 hour EC\textsubscript{50} value was observed, between any combination of test system (flasks or plates) and cell density surrogate (\textit{in vitro} or \textit{in vivo} Chl\textsubscript{a}) (Figure 4.9). This suggests that measurement of cell density in flasks and microplates is comparable. Furthermore higher NM300K sensitivity (lower EC\textsubscript{50} value) was observed in \textit{in vivo} methods compared to \textit{in vitro}, at all timepoints.

**Figure 4.9**: Impact of NM300K [30-150 µg/l] on \textit{R. subcapitata} growth rate inhibition at 72 hours in OECD medium, in flasks/\textit{in vivo} Chl\textsubscript{a} (black circles), flasks/\textit{in vitro} Chl\textsubscript{a} (grey squares) and 24 well plates/\textit{in vivo} Chl\textsubscript{a} fluorescence (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 2).
Table 4.4: Probit modelled effective concentrations of NM300K [30-150 µg/l] on *R. subcapitata* growth rate (24, 48, and 72 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model NM300K induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (*n* = 2). Superscript letters denote significant differences in the EC$_{50}$ value within test system, between each timepoint (One way ANOVA; *p* < 0.05); EC$_{50}$ values not sharing the same superscript letter were significantly different. No significant change in EC$_{50}$ value was observed over the test duration for 24 well plates *in vivo*.

<table>
<thead>
<tr>
<th>Method</th>
<th>24 hour</th>
<th>48 hour</th>
<th>72 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flasks <em>in vitro</em></td>
<td>44.87 (±1.57)*</td>
<td>80.41 (±1.71)*</td>
<td>117.21 (±1.80)*</td>
</tr>
<tr>
<td>Flasks <em>in vivo</em></td>
<td>30.79 (±1.84)*</td>
<td>57.58 (±1.99)*</td>
<td>94.86 (±1.64)*</td>
</tr>
<tr>
<td>24 well plates <em>in vivo</em></td>
<td>34.83 (±1.35)</td>
<td>50.90 (±3.12)</td>
<td>105.69 (±2.14)</td>
</tr>
</tbody>
</table>

AgNO$_3$ caused a concentration dependent increase in growth inhibition at 72 hours in both test systems (flasks and microplates). The time dependent increase in AgNO$_3$ EC$_{50}$ value observed previously in flasks (Section 3.5.1.) was detected across all three test designs (flasks (*in vitro* and *in vivo* Chl$_a$) and microplates (*in vivo* Chl$_a$)). For flasks, where *in vitro* Chl$_a$ was assessed, EC$_{50}$ values significantly increased over the test duration (*F* = 456.99, *p* < 0.001); rising from 1.84 (±3.11) µg/l at 24 hours to 4.87 (±0.11) µg/l at 72 hours. For the same test system, *in vivo* Chl$_a$ determined EC$_{50}$ values showed a similarly significant, but smaller increase over time (*F* = 61.67, *p* > 0.001); rising from 1.16 (±0.04) µg/l at 24 hours to 2.21 (±0.02) µg/l at 72 hours. Finally, a similar significant pattern of toxicity was observed for the 24 well plate test system, where EC$_{50}$ values estimated using *in vivo* Chl$_a$ (*F*$_{2,8}$ = 34.57, *p* = 0.001); rose from 0.71 (±0.03) µg/l at 24 hours to 2.56 (±0.10) µg/l at 72 hours (Table 4.5).

By 72 hours both *in vivo* Chl$_a$ derived AgNO$_3$ EC$_{50}$ values (Flasks 2.21 ±0.02 µg/l; Well plates 2.56 ±0.10 µg/l) were significantly lower than that obtained from flask based *in vitro* Chl$_a$ extraction (4.87 ±0.11 µg/l) (*F* = 132.58; *p* < 0.001) (Figure 4.10). Similarly to DCMU, *in vivo* Chl$_a$ shows a similar level of sensitivity between both flask and 24 well plate test designs, and is more sensitive than *in vitro* Chl$_a$ for detecting AgNO$_3$ toxicity. Unlike DCMU, all tests detected a similar increase in EC$_{50}$ over time.
Figure 4.10: Impact of AgNO₃ [0.2-20 µg/l] on *R. subcapitata* growth rate inhibition at 72 hours in OECD medium, in flasks/in vivo Chlₐ (black circles), flasks/in vitro Chlₐ (grey squares) and 24 well plates/in vivo Chlₐ fluorescence (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Table 4.5: Probit modelled effective concentrations of AgNO₃ [0.2-20 µg/l] on *R. subcapitata* growth rate (24, 48, and 72 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model AgNO₃ induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 1, one experiment with three replicates). Superscript letters denote significant differences in the EC₅₀ value within test system, between each timepoint (One way ANOVA; p < 0.05); EC₅₀ values not sharing the same superscript letter were significantly different.

<table>
<thead>
<tr>
<th>AgNO₃ EC₅₀ (µg/l Ag)</th>
<th>Method</th>
<th>24 hour</th>
<th>48 hour</th>
<th>72 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flasks <em>in vitro</em></td>
<td>1.84 (±3.11)³</td>
<td>2.21 (±0.06)²</td>
<td>4.87 (±0.11)¹</td>
</tr>
<tr>
<td></td>
<td>Flasks <em>in vivo</em></td>
<td>1.16 (±0.04)²</td>
<td>1.92 (±0.06)¹</td>
<td>2.21 (±0.02)¹</td>
</tr>
<tr>
<td></td>
<td>24 well plates <em>in vivo</em></td>
<td>0.71 (±0.03)³</td>
<td>1.54 (±0.05)²</td>
<td>2.56 (±0.10)³</td>
</tr>
</tbody>
</table>
The effect of lateral fluorescence from adjacent wells in clear 24 well plates was found to be minimal (Figure 4.11). A significantly high correlation was found between *in vivo* Chl$_a$ fluorescence, from *R. subcapitata* measured in clear plates immediately before transfer and re-measurement in black well plates ($r^2 = 0.98; p < 0.05$). This information can be used to inform design of future experiments (e.g. choice of test vessel).

![Figure 4.11 In vivo Chl$a$ data for three replicate experiments with *R. subcapitata* (NM300K dose response; both controls and toxicant levels included) assessed via 24 well plate. A positive correlation was observed between measurements made in black and clear 24 plates ($r^2 = 0.98; p < 0.05$) (n = 189).](image)

### 4.5.4. Humic acid and pH modification

The impact of humic acid and pH on the toxicity of both ionic and nanoparticulate silver was also tested to identify whether findings were comparable between flask and microplate test systems and measurement methods, under more complex media conditions. Only Ag (as NM300K and AgNO$_3$) was studied in this experiment.

In humic acid studies, a similar pattern of *R. subcapitata* control growth rate was observed in humic acid free cultures and cultures containing 5 mg/l SRHA (Figure 4.12). A significant difference in growth rate was observed between all three test designs in humic acid free cultures ($F = 77.72, p < 0.001$) and cultures containing 5 mg/l SRHA ($F = 49.90, p < 0.001$). In both test conditions, the highest growth rate was observed in flasks using *in vitro* Chl$_a$ (1.54 ± 0.03 and 1.47 ±0.08 d$^{-1}$, in the absence of humic acid and 5 mg/l humic acid respectively),
followed by flasks using *in vivo* Chl$_a$ (1.42 ±0.02 and 1.27 ±0.06 d$^{-1}$, in the absence of humic acid and 5 mg/l humic acid respectively), and finally 24 well plates using *in vivo* Chl$_a$ (1.11 ±0.02 and 1.16 ±0.06 d$^{-1}$, in the absence of humic acid and 5 mg/l humic acid respectively). For control cultures containing 50 mg/l SRHA, growth rate was also significantly different between all three test designs ($H = 16.10$, $p < 0.001$). However unlike humic acid free cultures/cultures containing 5 mg/l SRHA, the highest growth rate was observed in flasks using *in vitro* Chl$_a$ (1.24 ± 0.03 d$^{-1}$), followed by 24 well plates using *in vivo* Chl$_a$ (1.09 ±0.03 d$^{-1}$), and finally flasks using *in vivo* Chl$_a$ (0.97 ±0.03 d$^{-1}$) (Data not shown). In flask based methods (*in vitro* and *in vivo* Chl$_a$), a similar decrease in growth rate was observed with increasing humic acid concentration, as described in Section 3.5.2. However the same pattern of humic acid induced growth reduction was not observed in 24 well plates, which remained stable across all test conditions.

![Figure 4.12](image.png)

**Figure 4.12:** Experimental growth rate of *R. subcapitata* in control cultures of OECD medium over 72 hours in the presence and absence of Suwannee River humic acid (5 and 50 mg/l). Growth rate was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl$_a$ (black bars), flasks *in vitro* Chl$_a$ (grey bars) and flasks *in vivo* Chl$_a$ (white bars). Data expressed as mean growth rate and standard error of the mean ($n = 3$). Letters denote significant differences between methods within each humic acid condition (One way ANOVA/Kruskal-Wallis; $p < 0.05$); bars not sharing the same letter were significantly different.
The presence of SRHA reduced the toxicity of NM300K [190 µg/l] at 72 hours in a concentration dependent manner, in all test designs (i.e. flasks and microplates) (Figure 4.13). In the absence of SRHA, NM300K toxicity was significantly higher in flasks using *in vivo* Chl$_a$ ($H = 9.98$, $p = 0.007$); with a growth inhibition of 126.7 (±6.73), 104.67 (±5.25), and 96.82 (±4.34) % for flasks using *in vivo* Chl$_a$, flasks using *in vitro* Chl$_a$, and 24 well plates using *in vivo* Chl$_a$ respectively. This effect was different to NM300K concentration response studies, where no significant difference in 72 hour EC$_{50}$ value was observed between the three test designs (Figure 4.9 and Table 4.4). In the presence of 5 mg/l SRHA, NM300K mediated growth inhibition was 84.08 (±2.57), 92.63 (±5.91), and 100.28 (±4.31) % for 24 well plates using *in vivo* Chl$_a$, flasks using *in vitro* Chl$_a$ and flasks using *in vivo* Chl$_a$ respectively. However differences between test designs were not found to be significant for NM300K in the presence of 5 mg/l SRHA. In the presence of 50 mg/l SRHA, NM300K toxicity was significantly higher in flasks using *in vitro* Chl$_a$ ($H = 9.03$, $p = 0.011$), with a growth inhibition of 71.94 (±5.45), 53.7 (±4.05), and 51.71 (±2.83) % for flasks using *in vitro* Chl$_a$, flasks using *in vivo* Chl$_a$, and 24 well plates using *in vivo* Chl$_a$ respectively. Taken together, the data suggest slight differences in toxicity of NM300K between test designs in humic acid studies, but overall patterns of toxicity were retained. That is, NM300K toxicity was reduced by SHRA in a concentration dependent manner in all test systems.
Figure 4.13: Impact of NM300K [190 µg/l Ag] on R. subcapitata 72 hour growth inhibition in the presence and absence of Suwannee River humic acid (SRHA; 5 and 50 mg/l). Growth inhibition was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl$_a$ (black bars), flasks *in vitro* Chl$_a$ (grey bars) and flasks *in vivo* Chl$_a$ (white bars). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 2). Letters denote significant differences within each humic acid condition (Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different.

Both *in vivo* Chl$_a$ methods (flasks and 24 well plates) showed a similar SRHA concentration dependent reduction in growth inhibition following exposure to AgNO$_3$ (Figure 4.14), as observed for NM300K. The toxicity of AgNO$_3$ [6 µg/l] was not reduced in the presence of SRHA when growth inhibition was assessed in flasks using *in vitro* Chl$_a$ as a measure of growth inhibition as described in Section 3.5.2. (Figure 4.14). The only significant difference in AgNO$_3$ toxicity between test designs was observed in the absence of SRHA (F = 6.89, p = 0.028), with the highest growth inhibition observed in 24 well plates using *in vivo* Chl$_a$ (79.51 ±4.8 %), followed by flasks using *in vivo* Chl$_a$ (63.33 ±8.79 %), then flasks using *in vitro* Chl$_a$ (42.34 ±7.79 %). In the presence of 5 mg/l SRHA, AgNO$_3$ mediated growth inhibition was 59.40 (±6.17), 57.96 (±6.62), and 65.22 (±7.01) % for 24 well plates using *in vivo* Chl$_a$, flasks using *in vitro* Chl$_a$ and flasks using *in vivo* Chl$_a$ respectively. In medium containing 50 mg/l SRHA and AgNO$_3$, growth inhibition was 37.01 (±4.53), 39.09 (±2.31), and 31.18 (±3.08) % for 24 well plates using *in vivo* Chl$_a$, flasks using *in vitro* Chl$_a$ and flasks using *in vivo* Chl$_a$ respectively.
**Figure 4.14**: Impact of AgNO$_3$ [6 µg/l Ag] on *R. subcapitata* 72 hour growth inhibition in the presence and absence of Suwannee River humic acid (5 and 50 mg/l). Growth was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl$_a$ (black bars), flasks *in vitro* Chl$_a$ (grey bars) and flasks *in vivo* Chl$_a$ (white bars). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean ($n = 1$, one experiment with three replicates). Letters denote significant differences within each humic acid condition (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

In pH studies, *R. subcapitata* growth rate in unbuffered OECD medium was significantly different between all three test designs ($F = 167.30$, $p < 0.001$), with the highest growth rate observed in flasks using *in vitro* Chl$_a$ (1.55 ±0.02 d$^{-1}$), followed by flasks using *in vivo* Chl$_a$ (1.47 ±0.02 d$^{-1}$), and finally 24 well plates using *in vivo* Chl$_a$ (1.17 ±0.01 d$^{-1}$) (Figure 4.15). For toxicant free control cultures buffered to pH 6, 72 hour growth rate was only significantly lower in 24 well plates ($F = 143.51$, $p < 0.001$), with a growth rate of 1.11 (±0.02) d$^{-1}$, compared to 1.58 (±0.02) d$^{-1}$ in flasks using *in vitro* Chl$_a$ and 1.52 (±0.02) d$^{-1}$ in flasks using *in vivo* Chl$_a$. This pattern was the same in toxicant free control cultures buffered to pH 8; 72 hour growth rate was only significantly lower in 24 well plates ($H = 16.10$, $p < 0.001$), with a growth rate of 1.13 (±0.02) d$^{-1}$, compared to 1.52 (±0.02) d$^{-1}$ in flasks using *in vitro* Chl$_a$ and 1.48 (±0.01) d$^{-1}$ in flasks using *in vivo* Chl$_a$. In all test systems, pH condition (pH 6, pH 8) had no effect on *R. subcapitata* 72 hour growth rate compared to unbuffered medium, as described in Section 3.5.3.
Figure 4.15: Experimental growth rate of *R. subcapitata* in control cultures of OECD medium over 72 hours at a pH of 6 or 8. Growth rate was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chlₐ (black bars), flasks *in vitro* Chlₐ (grey bars) and flasks *in vivo* Chlₐ (white bars). Data expressed as mean growth rate and standard error of the mean (n = 3). Letters denote significant differences between methods within each pH condition (One way ANOVA/Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different.

A similar effect of pH on 72 hour growth inhibition following exposure to NM300K was observed between all test designs (Figure 4.16). NM300K increased toxicity in OECD medium buffered to pH 8 compared to unbuffered/pH 6 medium in all test designs, which aligns with previous studies conducted in flasks (Section 3.4.3). No significant differences in 72 hour growth inhibition were observed between test designs at any pH tested (Figure 4.16). In unbuffered medium NM300K mediated growth inhibition was 55.89 (±16.60), 58.86 (±11.37) and 82.11 (±10.85) % for 24 well plates using *in vivo* Chlₐ, flasks using *in vitro* Chlₐ and flasks using *in vivo* Chlₐ respectively. In medium containing NM300K and buffered to pH 6, growth inhibition was 91.68 (±25.56), 82.79 (±22.03), and 90.36 (±16.75) % for 24 well plates using *in vivo* Chlₐ, flasks using *in vitro* Chlₐ and flasks using *in vivo* Chlₐ respectively. In medium containing NM300K and buffered to pH 8, growth inhibition was 122.94 (±15.05), 122.44 (±12.75) and 131.97 (±8.26) % for 24 well plates using *in vivo* Chlₐ, flasks using *in vitro* Chlₐ and flasks using *in vivo* Chlₐ respectively.
Figure 4.1: Impact of NM300K [190 µg/l Ag] on R. subcapitata 72 hour growth inhibition at a pH of 6 or 8. Growth was estimated using different density surrogate methods/test systems; 24 well plate in vivo Chl\(_a\) (black bars), flasks in vitro Chl\(_a\) (grey bars) and flasks in vivo Chl\(_a\) (white bars). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each pH condition (One way ANOVA/Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different. No significant differences were found between tests designs.

Following exposure to AgNO\(_3\) [6 µg/l], toxicity in all test designs followed the same pattern of 72 hour growth inhibition, with a higher toxicity observed in OECD medium buffered to pH 8 compared to unbuffered/pH 6, as described in Section 3.5.3., and for NM300K above (Figure 4.17). The only significant difference in AgNO\(_3\) toxicity between test designs was observed in OECD medium buffered to pH 6 (F = 8.01, p = 0.02), with the highest growth inhibition observed in flasks using in vivo Chl\(_a\) (60.31 ±3.3 %), followed by flasks using in vitro Chl\(_a\) (51.21 ±2.85 %), then 24 well plates using in vitro Chl\(_a\) (41.40 ±3.80 %). In unbuffered medium containing AgNO\(_3\), growth inhibition was 40.62 (±3.88), 35.94 (±4.87), and 42.57 (±4.99) % for 24 well plates using in vivo Chl\(_a\), flasks using in vitro Chl\(_a\) and flasks using in vivo Chl\(_a\) respectively. In medium containing AgNO\(_3\) and buffered to pH 8, growth inhibition was 79.87 (±7.57), 94.43 (±5.46), and 101.65 (±4.60) % for 24 well plates using in vivo Chl\(_a\), flasks using in vitro Chl\(_a\) and flasks using in vivo Chl\(_a\) respectively.
Figure 4.17: Impact of AgNO$_3$ [6 µg/l Ag] on R. subcapitata 72 hour growth inhibition at a pH of 6 or 8. Growth was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl$_a$ (black bars), flasks *in vitro* Chl$_a$ (grey bars) and flasks *in vivo* Chl$_a$ (white bars). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). (n = 1, one experiment with three replicates). Letters denote significant differences within each pH condition (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Taken together the results from these studies demonstrate that all three toxicity test protocols show a high degree of similarity when testing NM300K and AgNO$_3$ toxicity in the standard OECD medium, in addition to a range of different medium conditions (presence/absence of humic acid, pH modification). In addition, slightly higher toxicity was observed (higher % inhibition) in 24 well plate tests than in flasks. A higher variability in growth inhibition was observed in flasks compared to microplates, and flask derived data from *in vivo* Chl$_a$ methods expressed higher % inhibition than *in vitro* Chl$_a$ methods.

Increasing concentration of SRHA reduced the toxicity (lower growth inhibition) of both forms of silver (ionic and nanoparticle), for both flasks and well plates, and across both approaches (i.e. *in vitro* and *in vivo* Chl$_a$) used to assess algal growth. Compared to unmodified OECD medium, a pH of 6 had no effect on toxicity of either form of silver. However at pH 8 a higher toxicity (higher % inhibition) was observed in both AgNO$_3$ and NM300K. Tests conducted in well plates showed a significant positive correlation with tests performed in flasks using *in vitro* Chl$_a$ for both humic acid ($r^2 = 0.78$, p < 0.001; Figure 4.18) and pH ($r^2 = 0.81$, p < 0.001; Figure 4.20) studies. Humic acid and pH studies show a similar pattern of results as
were observed in Section 3.5.1., where flask based *in vitro* Chl$_a$ methods were used to assess the growth of *R. subcapitata* and resulting toxicity of silver (i.e. a decrease in silver toxicity with increasing SRHA concentration, and an increase in silver toxicity at higher pH levels).

Growth inhibition data derived from *in vivo* Chl$_a$ methods showed a high correlation between both flask and well plate growth inhibition tests, especially in the case of SRHA ($r^2 = 0.84, p < 0.001$; Figure 4.20) which was higher than that observed when cell density estimation method (i.e. *in vivo* and *in vitro* Chl$_a$) was different between test systems (i.e. flasks using *in vitro* Chl$_a$ and well plates using *in vivo* Chl$_a$). In contrast, pH studies showed a similar correlation in terms of percent inhibition between flasks and well plates, regardless of density estimation method used ($r^2 = 0.88, p < 0.001$; Figure 4.19; $r^2 = 0.81 p < 0.001$; Figure 4.19). *In vivo* Chl$_a$ methods also showed a higher variability in flask based methods compared to microplates, which is particularly apparent in pH studies (Figure 4.19 and 4.21).

Finally, a good correlation was found between *in vivo* and *in vitro* Chl$_a$ methods of enumerating algal growth, in the same OECD standard flask based tests. Humic acid studies showed a stronger correlation ($r^2 = 0.94, p < 0.001$; Figure 4.22) than in pH studies ($r^2 = 0.88, p < 0.001$; Figure 4.23).
Figure 4.18: Comparison between *R. subcapitata* growth inhibition from 24 well plate test and the OECD standard method (conducted in flasks, *in vitro* Chl$_a$ extraction) for AgNO$_3$ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. 0 mg/l (black circles), 5 mg/l (grey squares) and 50 mg/l (white triangles) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vitro* Chl$_a$ and 24 well plates using *in vivo* Chl$_a$ (Pearson’s product-moment correlation, n = 81 total observations, $r^2 = 0.797$, p < 0.001). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.
Figure 4.19: Comparison between *R. subcapitata* growth inhibition from 24 well plate test and the OECD standard method (conducted in flasks, *in vitro* Chl*a* extraction) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent pH buffering (using 3.5mM 3-(N-morpholino)propanesulfonic acid), in toxicant free controls; i.e. unbuffered (black circles), pH 6 (grey squares) and pH 8 (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vitro* Chl*a* and 24 well plates using *in vivo* Chl*a* (Pearson’s product-moment correlation, n = 108 total observations, $r^2 = 0.810$, $p < 0.001$). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.
Figure 4.20: Comparison between *R. subcapitata* growth inhibition from 24 well plate test and the OECD standard method (conducted in flasks, *in vivo* Chl$_a$ extraction) for AgNO$_3$ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. 0 mg/l (black circles), 5 mg/l (grey squares) and 50 mg/l (white triangles) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vivo* Chl$_a$ and 24 well plates using *in vivo* Chl$_a$ (Pearson’s product-moment correlation, n = 81 total observations, $r^2 = 0.840$, p < 0.001). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.
Figure 4.21: Comparison between *R. subcapitata* growth inhibition from 24 well plate test and the OECD standard method (conducted in flasks, *in vivo* Chl*α* extraction) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent pH buffering (using 3.5mM 3-(N-morpholino)propanesulfonic acid), in toxicant free controls; i.e. unbuffered (black circles), pH 6 (grey squares) and pH 8 (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vivo* Chl*α* and 24 well plates using *in vivo* Chl*α* (Pearson’s product-moment correlation, n = 108 total observations, $r^2 = 0.882$, $p < 0.001$). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.
Figure 4.22: Comparison between *R. subcapitata* growth inhibition from two methods of measuring growth in the same OECD standard test system (flasks; *in vivo* and *in vitro* Chl$_a$) for AgNO$_3$ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. 0 mg/l (black circles), 5 mg/l (grey squares) and 50 mg/l (white triangles) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vitro* Chl$_a$ and flasks using *in vivo* Chl$_a$ (Pearson’s product-moment correlation, $n = 81$ total observations, $r^2 = 0.940$, $p < 0.001$). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.
Figure 4.23: Comparison between *R. subcapitata* growth inhibition from two methods of measuring growth in the same OECD standard test system (flasks; *in vivo* and *in vitro* Chl) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent pH buffering (using 3.5 mM 3-(N-morpholino)propanesulfonic acid), in toxicant free controls; i.e. unbuffered (black circles), pH 6 (grey squares) and pH 8 (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vitro* Chl, and flasks using *in vivo* Chl (Pearson’s product-moment correlation, \(n = 108\) total observations, \(r^2 = 0.879, p < 0.001\)). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.

4.6. Discussion

The primary aims of this chapter were to assess the suitability of using three surrogate methods of determining algal cell density. In the first instance the suitability of using absorbance, *in vivo* Chl, *in vitro* Chl, to measure algal cell density in flasks was assessed via comparison to the performance of manual cell counts. As such, the hypothesis that surrogate measurements of cell density will show a high correlation with cell counts was supported.

Next, the suitability of applying these alternative approaches to measure cell density in miniaturised (24 well plate) toxicity tests was investigated, with the aim of making testing more rapid and reliable. A range of chemical substances (AgNO₃, NM300K, and DCMU) and media (standard OECD, and standard OECD with pH and SRHA modifications) were tested to assess its variability across a range of conditions.
All three cell density surrogate methods showed a highly significant correlation with manual cell counts in the following order: absorbance > *in vivo* Chl$_a$ > *in vitro* Chl$_a$. Despite absorbance showing a slightly higher correlation with manual counts, *in vivo* Chl$_a$ was identified as the preferred method of cell density quantification, due to its ease of integration with small volume (1.5 ml) well plate tests. Optical methods required too high a volume to detect low densities of algal cells (ca. 25 ml per sample), and thus could not be applied to a microplate set up. Furthermore, as *in vitro* Chl$_a$ methods required a destructive sampling regime of a minimum of 1 ml per sample per day, it could not be measured using microplate tests. Whilst *in vitro* Chl$_a$ was not suitable for 24 well plates, it is still sensitive enough to be used in flask based studies (Chapters 3 and 5) where sample volume is of lesser concern.

Having identified a suitable measurement method for use in the microplate toxicity assay, the sensitivity of the miniaturised tests was compared to the standard OECD growth inhibition test guideline using flasks (OECD, 2011). Whilst *R. subcapitata* growth rate was compared between *in vivo* Chl$_a$ methods for both microplates and flasks, *in vitro* Chl$_a$ methods could only be applied to flasks due to the destructive sampling required (as described above). Test designs were compared across concentration response data (DCMU, AgNO$_3$, and NM300K) to identify the impact of test conditions on the EC$_{50}$ values for the toxicants. In addition, the influence of SHRA and modifications in pH on silver (ionic and nanoparticle) toxicity were investigated. The results obtained suggest that a miniaturised OECD 201 algal growth inhibition test (microplates) can allow for a more rapid assessment of determining cell density, and provides comparable findings to those obtained using the standard flask based test design (*in vivo* Chl$_a$).

### 4.6.1. Selection of methods to estimate cell density

The aim of this work was to find a suitable surrogate method for manual cell counts, which had potential application to determining algal growth in miniaturised, microplate test system. Manual cell counts are time consuming, and have high variability both within and between investigators. Whilst electronic particle counters are available, their high cost prohibits their widespread use (Geis et al., 2000). Provided the conversion factor between the surrogate parameter and manual cell count is known, the application of surrogate methods satisfies the requirements of the OECD 201 test (OECD, 2011). If the relationship between the surrogate measure of cell density and cell count data is linear over the test duration, growth inhibition data can be obtained from unconverted surrogate measurements (as was done in this thesis), as the test standardises growth rate to percentage of control. When a stationary phase, high cell density algal culture (ca. 6-8 x 10$^6$ cells/ml) was serially diluted, and
correlations between surrogate measurements and manual cell counts were made, the
relationship between the surrogate methods and cell counts were higher than that of actively
growing cultures (the later presented in this chapter). However the use of actively growing
cultures in this work was considered to be more relevant, as any potential changes in *R. subcapitata* Chl, content/cell morphology over the growth period could be accounted for. For
example, Griffiths *et al.* (2011) found that estimations of cells density using absorbance were
affected by changes in pigment concentration over the growth curve of *C. vulgaris*,
*Scenedesmus spp, Spirulina platensis* and *Nannochloropsis spp*. As a result of rapid exponential
growth, there is a notable data gap between cell counts of 2 and 5 x 10⁶ cells/ml. This could
have been addressed by increased sampling frequency during the exponential growth period.
It is likely that there is a biphasic change in cellular Chl, content over the growth period, with
an increase in Chl, content per cell during exponential growth, relative to a decrease in
stationary phase cultures, which has been observed for other freshwater algal species
(Griffiths *et al.*, 2011). However, as the OECD 201 test guideline requires cultures to be at
exponential growth over the test duration, errors associated with changes in pigment
concentration should theoretically be minimised.

Photometric (absorbance) methods of assessing algal growth have been criticised by
previous authors due to their low sensitivity, especially when compared to fluorescent
measurements of cell density (Eisentraeger *et al.*, 2003; Kvíderová, 2009; Griffiths *et al.*, 2011;
Van Wagenen *et al.*, 2014). Sensitivity of photometric methods can be improved when
assessing algal growth in flasks through the use of 10 cm path length cuvettes in a
spectrophotometer (OECD, 2011), and determining absorbance at 685 nm (*c.f.* 430 nm;
Environment Canada, 1992) which has lower potential for interference with other algal
pigments (Geis *et al.*, 2000; Rojíčková *et al.*, 1998). These were sufficient for quantifying algae
cultures to a density required for standard OECD tests (5 x 10⁴ cells/ml) in this study, and
became standard practice for preparing pre-inoculum cultures of algae at the beginning of
each test. However at higher absorbance (> 1) it was necessary to dilute algal cultures, or
reduce the cuvette path length to 1 cm to measure cell density. It is suggested that monitoring
of algal growth via absorbance is not sufficiently reliable to derive accurate EC₅₀ values
(Eisentraeger *et al.*, 2003), and thus *in vitro* Chl, was adopted as the preferred method in this
study. Absorbance has been used to determine toxicity in microplates for other algal species
(Kvíderová, 2009; Ribeiro *et al.*, 2015; Golubev et al., 2016), but due to the high growth rate of
*R. subcapitata* in flasks and the need for dilution, optical density was not selected as a
candidate for estimating growth.
In vitro Chl\textsubscript{a} is a fluorescence based, commonly used surrogate for measuring algal cell density in many studies. In order to measure Chl\textsubscript{a} content using this method, it first has to be extracted from algal cells. Adaptations of the “whole water” extraction method (i.e. no requirements for filtration of algal cultures) proposed by Mayer et al. (1997) have been used in a number of algal toxicology studies (Halling-Sorensen et al., 2000; Mulderij et al., 2003; Arujoa et al., 2004; Christensen et al., 2006; Hartmann et al., 2010; Van Hoecke et al., 2013; Sorensen and Baun, 2015). Mayer et al. (1997) suggests the use of a 1:1 solution of dimethyl sulfoxide (DMSO) and acetone, for optimal Chl\textsubscript{a} extraction in 20 minutes. Adaptations to the original method in this thesis included the addition of locust gum to precipitate out nanoparticles in order to avoid potential nanoparticle interference with fluorescence measurements (Kinross, unpublished data), and the extraction in acetone only in order to simplify the extraction procedure. The use of acetone only however, extended the required extraction time to ca. 3 days (Kinross, unpublished data). Furthermore, measurement of extracted samples is more time consuming than other methods which don’t require extraction (in vivo Chl\textsubscript{a}). Growth rates measured using in vitro Chl\textsubscript{a} were significantly lower than those observed for cell counts, and correlations between these two methods were the lowest. However, comparable toxicity data could be generated since growth rate inhibition is determined in relation to the respective control, in the same experimental conditions. Comparisons of cell count and in vitro Chl\textsubscript{a} fluorescence-derived toxicity of K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} to \textit{R. subcapitata} have demonstrated the two methods generate very similar data, at all concentrations which induce < 80 \% growth inhibition, for 72 hour exposures (Mayer et al., 1997). Despite this method’s suitability for standard growth inhibition tests (it was used to successfully generate concentration response data in Chapter 3, and as the established method in other algal toxicology work at Heriot Watt University) its requirements for small volumes of sample to be permanently removed for pigment extraction disqualify it as a potential candidate for miniaturisation. The 1 ml volume used for in vitro Chl\textsubscript{a} extraction was primarily selected to ensure adequate sample volume for measurement in the fluorimeter, and to allow any nanoparticles to settle out with the aid of locust gum. However Aruoja et al. (2004) developed an extraction method using only 50 µl of algal culture, which show potential for use in future miniaturised studies, as the extraction method presented in this thesis required too large a volume.

In vivo Chl\textsubscript{a} is a rapid method of quantifying growth in algal studies, which has been demonstrated to be more sensitive than measurements of growth using absorbance (Geis et al., 2000; Eisentraeger et al., 2003; Van Wagenen et al., 2014), and more rapid than assessment of growth using in vitro Chl\textsubscript{a}. As with in vitro methods, in vivo Chl\textsubscript{a} selectively
quantifies the signal of a single photo-pigment, which makes it suitable for quantifying algae in both environmental samples containing humic substances (Gregor and Maršálek, 2004) as well as laboratory prepared suspensions containing nanoparticles (Lee and Ann, 2013; Hund-Rinke and Simon, 2006), both of which may interfere with optical density readings (Kinross, unpublished data). In addition, the results here suggest in vivo Chlₐ can be used to predict cell count derived cell density with slightly greater accuracy than in vitro Chlₐ. Its utility as a non-destructive technique is not only valuable for quantifying cell density in microplates, but also has the potential to reduce the required test volume for OECD standard, flask based toxicity tests.

4.6.2. Control culture performance in flasks and well plates

Measurement of in vivo Chlₐ was identified to be the most suitable method for quantifying cell density in miniaturised toxicity tests, due to its high sensitivity and non-destructive sampling. The sensitivity of the different approaches to assess R. subcapitata growth rate could be ranked as follows: flasks using in vitro Chlₐ > flasks using in vivo Chlₐ > 24 well plate in vivo Chlₐ. Whilst this does not support the hypothesis that control growth rate will be the same across test design, the 72 hour growth rate in all test designs was always higher than 0.92 d⁻¹ – the minimum required growth rate for OECD 201 test validity (OECD, 2011), which suggests that the test can be miniaturised whilst still satisfying this test requirement. Eisentraeger et al. (2003) found similar growth rates for algae in 24 well plates when compared to flasks, but only when increasing rotational speed of well plates using a shaker.

Flask based toxicity tests generally resulted in a higher variability than in 24 well plates, regardless of whether in vivo or in vitro Chlₐ methods were used to assess cell growth. Arensberg et al. (1995) hypothesised that during incubation, miniaturised tests should experience less light variation than flasks, due to the smaller incubation space required. The light distribution within the incubator used in these studies varied by approximately 10 %, which may account for flask based tests showing higher variation. Flasks were replaced randomly in the shaker each time a measurement was carried out (in accordance with OECD, 2011), whereas well plates were always fixed in a single location. Geis et al. (2000) rotated each well plate by 90° each day, which may also serve to reduce growth variation induced by uneven lighting. It is therefore recommended that when using well plates, growth variation can be minimised without the need for moving/rotating well plates, unless a high variation in light level is suspected across the incubator (however using such incubators should not be considered good laboratory practise). In addition, randomisation of treatments in wells between daily readings may be employed in a similar manner to OECD 201 protocol for flasks.
(OECD, 2011), and may prevent edge effects from evaporation. However if the aim of well plate test designs is to increase testing efficiency, such a measure may be counterproductive.

*In vivo* Chl$_a$ appears to be a superior method for determining algal growth in toxicity tests, due to the better correlation with manual cell count, lower sample processing time, and more sensitive estimates of toxicity for the toxicants studied. Regardless of whether flasks or plates were used, this method could be easily deployed and results were obtained rapidly. In the case of flask based *in vitro* Chl$_a$ methods, volumes as small as 200 µl can be used to reliably estimate cell density, further reducing required test volume.

Maintaining a pH within the limit permitted by the OECD is important. As demonstrated in Chapter 3, a pH change of < 2 units can have significant effects on toxicity estimations, and control of pH change over the test duration should be of high priority during algal toxicity tests. Whilst measurement of pH can be easily monitored in flasks, it is more challenging to monitor the pH of algal cultures in small volumes, such as 24 well plates. In algal cultures, the surface area to volume ratio is thought to exert a strong influence on CO$_2$ drawdown and therefore offsetting the tendency of algae to increase pH as a result of carbon uptake (Arensberg *et al.*, 1995). When flasks contain 50 ml of liquid (as in the standard OECD 201 test), the surface area of the test medium exposed to the air corresponds to approximately 58 cm$^2$. A volume of 50 ml provides the optimal surface area to volume ratio in 250 ml flasks due to the conical shape, whilst at the same time providing enough media for daily removal of samples to monitor growth (e.g. *in vivo/in vitro* Chl$_a$ and cell counts). In 24 well plates, medium surface area remains constant at 1.88 cm$^2$, irrespective of volume. Based on the volumes used in these tests (1.5 ml in 24 well plates and 50 ml in flasks), surface area to volume ratio corresponds to 1.25 in wells and 1.16 in flasks. Although the available pH electrode was too large to measure pH in 24 well plates without affecting test volume, with equal shaking speed and light intensity, one may predict that a higher surface area to volume ratio resulted in a sufficient CO$_2$ buffering of well plate test systems. A similar result was found by Arensberg *et al.* (1995), where higher surface area to volume ratio in miniaturised toxicity tests (1.96) resulted in greater pH stability compared to the same flask design as used in these studies (though the authors of this paper claimed this effect was due to poor shaking of flasks). Whilst this finding is not conclusive, future studies should ensure measurement of pH during the test using micro pH meters, designed specifically for measuring pH in well plates.

A number of authors have chosen 24 well plates for miniaturisation work (Geis *et al.*, 2000; Lee and An, 2013), with some explicitly preferring them over 96 well plates (Geis *et al.*, 2000). Attempts were made in this work to develop a reliable 96 well algal toxicity microassay,
but failure to reliably do so was attributed to poor CO\textsubscript{2} drawdown in the early stage of the test and evaporation at later stages (data not shown). The future development of 96 well assays is possible, with the main obstacle being the trade-off between needs to prevent evaporation whilst simultaneously allowing adequate gas exchange. This obstacle may be overcome by the development of humid/CO\textsubscript{2} enriched test chambers specifically designed for high throughput toxicity testing with algae. Such test chambers are not currently widely available, and the 24 well plate study was chosen in this chapter due to its suitability for use in the test chambers used to incubate flasks. Plates used in this study were sealed to prevent evaporation (observed in pilot experiments with unsealed plates), however this may have limited gas exchange which may have explained the lower growth rate observed in well plates relative to flasks. Parafilm was chosen to seal plates on the basis that it is reportedly gas permeable (Eisentraeger et al., 2003; Riedl and Altenburger, 2007), however these results may call this into question (as have Van Wagenen et al., 2014). The use of such films must be carefully considered as a potential source of variation in studies seeking to reproduce the findings presented here.

There are currently a number of advantages to 24 well plates relative to 96 well plates including the use of a larger sample volume (lower susceptibility to evaporative effects), and the ability to effectively shake these plates in incubators usually used for flasks. Fluorescence can be measured in clear or black well plates. The use of black plates is often preferred as it optimises signal to noise ratio in the plate reader, by preventing the fluorescent signal in adjacent well interfering with the reading. In this work it was demonstrated that there was no requirement to use black plates as fluorescence readings were comparable in clear 24 well plates and black 24 well plates. This is advantageous as the transfer of samples to black 24 well plates for fluorescence readings, requires considerably more handling time compared to clear 24 well plates, which can be read directly (Eisentraeger et al., 2003).

4.6.3. Toxicity estimates between methods

For DCMU, most published studies focus on photosynthetic inhibition as the standard endpoint to evaluate toxicity (Ralph et al., 2007; Chapter 5). Published studies on DCMU mediated growth inhibition of \textit{R. subcapitata} at 72 hours are rare, but EC\textsubscript{50} values range from 6.6-17.2 µg/l at 25°C (Okamura et al., 2003; Tasmin et al., 2014). The effect of DCMU on \textit{R. subcapitata} growth inhibition decreases both with increasing temperature (10-30 °C) and time (72-144 hours) (Tasmin et al., 2014). In 24 well plates using \textit{in vivo} Chl\textsubscript{a}, DCMU EC\textsubscript{50} values are comparable with published studies (e.g. Tasmin et al., 2014), with a small but significant decrease in toxicity observed between 24 and 72 hours. However in flasks using \textit{in vitro} Chl\textsubscript{a} as a measure of algal growth, toxicity was both lower than in well plates, and significantly
increased over the same time period (see Appendix 4 for full toxicity results in standard OECD test). In flasks using *in vivo* Chl*α*, the DCMU 72 hour EC₅₀ value was not significantly different than in well plates. These results indicate that surrogate cell density measurement may affect estimates of toxicity more than vessel type for this toxicant. Interestingly, Tasmin *et al.* (2014) also used *in vivo* Chl*α* to assess density, but the exact measurement method (e.g. excitation/emission wavelengths) was not described.

*In vivo* Chl*α* methods of determining growth inhibition, in both flasks and well plates, were significantly more sensitive as indicated by lower EC₅₀ values than *in vitro* Chl*α* methods (flasks only) for DCMU and ionic silver. More specifically, the *in vivo* 72 hour EC₅₀ value was approximately half the *in vitro* 72 hour EC₅₀. In the case of nanosilver, the three test designs showed similar sensitivity with respect to 72 hour EC₅₀ estimates (i.e. EC₅₀ was not significantly different). The increased sensitivity of *in vivo* Chl*α* methods at estimating cell density was also supported by the SRHA and pH studies with ionic and nanoparticulate silver. Such increased sensitivity of *in vivo* Chl*α* may be related to the fact that it is a direct measurement of Chl*α*, whereas *in vitro* Chl*α* requires processing of the sample which may decrease sensitivity (e.g. acetone extraction). Given this finding, the hypothesis that EC₅₀ will be similar between test systems was only supported in the case of NM300K, but not AgNO₃ or DCMU. Extracted *in vitro* Chl*α*, originally proposed by Mayer *et al.* (1997) has been used and adapted by many studies investigating toxicant-induced algal growth inhibition (Halling-Sorensen, 2000; Mulderij *et al.*, 2003; Arujoa *et al.*, 2004; 2009; Christensen *et al.*, 2006; Hartmann *et al.*, 2010; Van Hoecke *et al.*, 2013; Sekine *et al.*, 2015; Sorensen and Baun, 2015). A smaller number of growth inhibition studies have been performed using *in vivo* Chl*α* methods (Eisentraeger *et al.*, 2003; Hunde-Rinke and Simon, 2006; Lee and An, 2013; Sekine *et al.*, 2015). These results have important implications for comparing toxicity data between studies, as they suggest that algal density enumeration method may have a greater effect on EC₅₀ estimates than toxicant test chamber choice (i.e. flasks *cf.* well plates), for the toxicants considered here.

Fluorometric methods of assessing cell density (Hunde-Rinke and Simon, 2006; Lee and An, 2013) are advantageous over both photometric methods and manual cell counts as they are more sensitive, less laborious and less variable. Similar fluorometric protocols have been used to successfully assess the toxicity of Ag (Sekine *et al.*, 2015), TiO₂ (Hunde-Rinke and Simon, 2006; Lee and An, 2013) and ZnO (Lee and An, 2013) nanoparticles to algae. In addition, *in vivo* fluorescence can be used with turbid environmental samples (Eisentraeger *et al.*, 2003). Humic acid can interfere with fluorescence readings but this was overcome by the use of cell free SRHA blanks (at 5 and 50 mg/l). The signal from these blanks remained unchanged over the test duration, and interfering fluorescence was additive so could easily be subtracted from
samples containing humic acid and algae. The same approach was used for silver (NM300K and AgNO₃) and OECD medium buffered to pH 6 or 8. Unlike SRHA, these cell free blanks did not exhibit any additional fluorescence compared to unmodified OECD medium. It is worth noting that the in vivo Chlₐ method used at Heriot Watt was originally developed for use with TiO₂ nanoparticles, due to their interference with absorbance methods (Kinross, pers. comms.). Identifying potential fluorescence interference of a variety of nanomaterials was outside the scope of this work, but represents a significant barrier to the adoption of such methods to other nanomaterials. In cases where the fluorescence nanomaterial blanks are demonstrated, the in vitro Chlₐ method presented here may be preferable, due to the addition of locust gum which aids in the precipitation of suspended nanomaterials.

Although the development of miniaturised algal growth systems has been occurring in parallel to the use of OECD 201 test, the more widespread use of miniaturised methods have not received much attention from a regulatory perspective. Whilst OECD 201 (2011) does not explicitly state that miniaturised methods such as well plates cannot be used, it does state that flasks are the typical test vessel. This may be related to requirements for measuring pH and the previous requirement to use invasive methods of enumerating growth which resulted in inevitable sample consumption. Indeed guidelines explicitly state that “small volumes removed from test solution by pipette” for sampling must not be returned to the test vessel. In recent years, the development of non-invasive methods of enumerating cell density (Fai et al., 2007) and microplate pH meters (Yamada et al., 2010) have reduced the required volume. CO₂ is a rate limiting gas for algal growth (as a substrate for photosynthesis) and a lack thereof causes an increase in medium pH and lowers algal growth rate (Arensberg et al., 1995). As such, the slightly reduced growth rate observed in 24 well plates may indicate a higher pH (though it should be noted that OECD medium has a good buffering capacity due to its NH₄⁺ content – see Chapter 2). The pH meter available was too large to adequately measure pH in this work, but future studies should try to measure pH as OECD guidelines stipulate (OECD, 2011).

Overall, these results show a good correlation between standard (flask) and miniaturised (24 well plate) assays, especially when the same method is used to determine cell density (in vivo Chlₐ). This supports previous literature comparing standard and miniaturised toxicity tests for algae (Arensberg et al., 1995; Rojíčková et al., 1998; Geis et al., 2000; Okamura et al., 2002; Eisentraeger et al., 2003; Paixão et al., 2008), and suggests the possibility that miniaturised toxicity tests can be used to screen the wide variety of new nanomaterials currently in development. Whilst further work may be necessary to identify broad scale suitability, the ease of simultaneous deployment of standard and miniaturised tests has been highlighted by this research. Well plate tests were developed to use the same
test conditions (temperature, light regime, shaking speed) as standard tests, and enumerating cell density requires no additional equipment. It is therefore recommended that laboratories which have the capacity to run such toxicity tests in parallel do so as a matter of routine; as such data will aid the further development of this research area at little extra cost. Ultimately it is suggested that the use of miniaturised systems replaces flask based methods for the reasons previously stated, however more validation is needed in order for such methods to become incorporated into the OECD 201 test guideline.

Silver adsorption concerns, the role of the test vessel

The toxicity (growth inhibition) of AgNO$_3$ and silver nanoparticles to *R. subcapitata* presented in this chapter is comparable to the literature (Bondarenko *et al.*, 2012; Sekine *et al.*, 2015). Both plastic (polystyrene) and glass have been demonstrated to adsorb silver nanoparticles (Malysheva *et al.*, 2015; Sekine *et al.*, 2015), the extent to which this occurs is likely dependent on particle properties (coating, size, etc.) as this controls their interaction with media and the test vessel. Low silver nanoparticle adsorption to polystyrene well plates compared to glass has been attributed to steric stabilisation (Malysheva *et al.*, 2015). In this chapter, the NM300K EC$_{50}$ value did not significantly differ between glass flasks and polypropylene well plates. This observation may be due to the good dispersion of NM300K in algal media due to the presence of non-ionic polymeric stabilisers (polyoxyethylene (20) sorbitan mono-laurat and polyoxyethylene glycerol trioleate; see Section 2.1.1. for more detail). The toxicity of NM300K and AgNO$_3$ decreased over time (as indicated by a higher EC$_{50}$ value at later time points). Sekine *et al.* (2015) noted a similar time dependent reduction in AgNO$_3$ toxicity in 20 ml glass vials, and attributed this to ionic silver adsorption to the test vessels. The authors also found that polypropylene tubes (i.e. the same material as the well plates used in this study) adsorbed very little ionic silver over 72 hours compared to glass flasks. They reasoned (but did not test) that such plastic test containers may help ensure constant ionic silver exposure conditions and prevent the time dependent reduction in toxicity observed in glass. In the data presented here however, both glass flasks and polypropylene well plates showed a significant reduction in AgNO$_3$ toxicity over time. Although adsorbed silver was not measured in this work, given the findings of Sekine *et al.* (2015) it is likely that the reduction in toxicity is not solely a consequence of silver adsorption to the test vessel and that other mechanisms are responsible for this (e.g. algal removal through the production of exudates or biochemical detoxification; see Section 3.6.6. for more detail).

The use of plastic vessels in bioassays is an often mentioned concern, and allegedly one of the most often cited objections to adoption of algal microassays. For example,
McDonald et al., (2008) found that compounds such as oleamide can leach from plastics and inhibit the efficacy of some bioassays. However such findings have wider implications for other routinely used plastic ware (e.g. petri dishes). The fact that many authors in the field of ecotoxicology already use plastic vessels (Fortin and Campbell, 2001; Lee et al., 2004; 2005; Hiriart-Baer et al., 2006; Navarro et al., 2008; Chen et al., 2013), calls the validity of such concerns into question. With some authors using glass and others using plastic, it is important to know to what extent toxicology data for different substances generated in one can be applied to another (Eisentraeger et al., 2003; Geis et al., 2000). This study has demonstrated that the method of cell density estimation is more influential than vessel material, for DCMU AgNO₃ and NM300K.

4.7. Conclusions

This work shows that a number of commonly used surrogate methods of determining algal cell density can be successfully used to determine *R. subcapitata* growth rate, and so be used as a time-saving method to conduct algal growth inhibition assays. Furthermore the data presented here adds additional support to the potential development of a standardised miniaturised OECD 201 Algal Growth Inhibition Test using *R. subcapitata*. Results comparing control culture growth rate and toxicity estimates (EC₅₀) for NM300K were similar between both the standard method in flasks and the miniaturised method 24 well plates under the same incubation conditions. However for DCMU and AgNO₃, 72 hour growth inhibition EC₅₀ was lower when measured using *in vivo* Chlₐ fluorescence, regardless of whether the tests themselves were conducted in 250 ml borosilicate flasks or polypropylene 24 well plates. This work has shown that laboratories equipped to perform flask-based, standard OECD 201 tests have adequate opportunity to conduct well plate cross-validation studies of their own at little extra cost. Such data will be increasingly valuable in order to determine the sensitivity of miniaturised methods to a range of common and novel toxicants. With registries like the Chemicals Abstracts Service (CAS) entering an increasing number of nanomaterials and other substances into their databases, the number of laboratories able to assess their risk should not be prohibitive to understanding their toxicology, and the progression of the industry as a whole.

The ability to use fluorometric methods to provide a surrogate measure of algal cell density has facilitated the development of miniaturised tests. The ease of measurement conferred by such methods lends itself to potential future automation of this test with high throughput screening in mind. As sampling is not invasive, no sample volume is used up with repeated measurements. This allows excellent temporal data resolution through increased
measurement frequency, which may be useful in situations where short term dynamic changes in test concentration/speciation may be suspected. Furthermore, the specificity of fluorometric methods allow them to be used in samples containing humic substances, potentially allowing a battery of environmentally relevant samples to be included in such test regimes.

Improving the speed/reproducibility of standard ecotoxicological tests is essential if regulation is to keep pace with industry. Given that a single well plate typically occupies the same space as two 250 ml Erlenmeyer flasks, a standard 24 flask exposure occupies the same amount of space as 288 wells, or 12 24-well plates. Furthermore that number of wells uses the same volume (assuming 1.5-2 ml per well) of potentially toxic medium as 11 standard flasks (assuming 50 ml per flask). As such a given laboratory can greatly increase the replication/resolution of its toxicity data, whilst at the same time minimising its own potentially toxic output.

Further work is required to harmonise protocols, and fully understand the potential interaction of different toxicants with well plate based tests systems, including nanomaterials. A potential SOP for future miniaturised OECD 201 testing is presented in Appendix 5. Concerns over the suitability of existing test protocols for the assessment of nanomaterial toxicity, are just as valid to the development of new test methods (if not more so). Answering such questions is crucial to understanding the suitability of such tests in the future and their value to industry and the regulatory process.
Chapter Five - Photosynthetic efficiency as a sublethal endpoint

5.1. Introduction

The OECD 201 test, which assesses toxicity of a substance to algal growth are typically conducted over 3 days, with the shortest recommended time point normally being 48 hours (OECD, 2011; Chapter 3). Given the growing production of novel nanomaterials, there is a high regulatory demand for reduced testing durations, to enhance the efficiency of nanotoxicology testing. The advantages of improved efficiency include rapid acquirement of toxicity data, testing of a greater number of substances in a shorter amount of time, and improved robustness of data through increased replication. It is established that silver ions and nanomaterials may undergo transformative processes not only in the environment (McLaughlin and Bonzongo, 2012) but also over the duration (i.e. hours to days) of a standard toxicity test (Kittler et al., 2010; Chappell et al., 2011; Pettitt and Lead 2013; Sorensen and Baun, 2015; Chapter 2.5). Short term (< 24 hours), sub-lethal tests may offer an alternative to existing algal toxicity tests, especially when nanomaterial behaviour is unstable over the duration of the test. By testing over shorter time periods (minutes/hours cf. days), the effect of nanomaterial behaviour on toxicity may be reduced or avoided (Petersen et al., 2014; Sorensen and Baun, 2015). Although short term tests may not be truly representative of environmental processes, they may serve as a robust regulatory tools, as a variety of toxicants can be screened more rapidly. However, for such tests to be of value, they must first be demonstrated to be sensitive and correlate with the established, longer term, standard tests. Microbial species are particularly suitable for short term, high-throughput methods.

Primary producers such as algae play a fundamental role in aquatic ecosystems by fixing carbon and forming the base of the food web. It therefore follows that toxic impacts to these species may have deleterious effects at higher trophic levels (Kalman et al., 2015), especially in the case of silver (Bondarenko et al., 2013). The majority of current microalgal toxicity assays use growth inhibition as the primary endpoint when assessing toxicity, and a number of standard test guidelines have been developed for this purpose (ISO, 2006; OECD 2011). As Chapter 3 describes, such tests can be reliably used to estimate the EC50 value of silver nanoparticles, and others have demonstrated its use with other nanomaterials (Baun et al., 2008; Aruoja et al., 2009; Sadiq et al., 2011).

Algae’s unique ability to fix carbon through photosynthesis offers a number of unique endpoints for assessment of toxicity (Miles, 1990; Ralph et al., 2007; Oukarroum et al., 2012) which cannot be studied in animal/bacterial/fungal models. Inhibition of various
photosynthetic processes has been used as an indicator of toxicity for a wide range of substances (e.g. industrial chemicals, pharmaceuticals, herbicides). As such, photosynthetic inhibition has been suggested as an alternative to the standard algal growth inhibition assay (Petersen and Kusk, 2000; Radix et al., 2000; Nestler et al., 2012) and as an endpoint to monitor the effect of toxicants in natural bodies of water (Muller et al., 2008; Peña-Vázquez et al., 2010; Sjollema et al., 2014a). Environmental photosynthetic biosensors containing immobilised cyanobacterial proteins have also been developed which can be used to detect photosynthetically active herbicides (Koblizek et al., 2002; Maly et al., 2005; Masojidek et al., 2011). Through the use of fluorometry, a variety of photochemical endpoints can be studied rapidly and non-destructively (Ralph et al., 2007; Table 5.1). It is clear that a suite of short term (< 24 hour) photosynthetic parameters have the potential to inform broad-scale hazard assessment for a range of toxicants, yet a lack of a clear standard protocol has been suggested by some authors as one of the main impediments to the regulatory adoption of such endpoints (Ralph et al., 2007). For metals, the majority of studies have focussed on copper (e.g. Dewez et al., 2005; see Barón et al., 1995 for review), but other studies have also demonstrated the photoinhibitory effect of metals such as zinc (Prasad and Strzalka, 1999), lead (Miles et al., 1972), cadmium (Prasad and Strzalka, 1999; Faller et al., 2005) and chromium (Ali et al., 2006). In nanomaterial ecotoxicology research, zinc (Tang et al., 2013), copper (Saison et al., 2010), gold (Perrault et al., 2012; Behra et al., 2015) and silver (Navarro et al., 2008; 2015; Miao et al., 2009; Dewez and Oukarroum, 2012; Oukarroum et al., 2012; Matorin et al., 2013) nanomaterials have all been demonstrated to exhibit concentration dependent effects on algal photosynthetic processes. Whilst such studies are useful, differences in methodology (e.g. media composition, microalgal species, exposure period) prevent meaningful comparisons of results. Navarro et al. (2015) found that differences in silver nanoparticle coating and size had little effect on 1 and 2 hour photosynthetic efficiency inhibition (ΦPSII, Table 5.1) in C. reinhardtii. Dewez and Oukarroum (2012) studied the effect of 50 nm silver nanoparticles on a variety of photosynthetic parameters in C. reinhardtii, after 3 and 6 hours of exposure in dark and light conditions and demonstrated that silver nanoparticle toxicity increased over time (this effect was further enhanced in light conditions). Oukarroum et al. (2012) found that photosynthetic toxicity of the same silver nanoparticles (Dewez and Oukarroum, 2012) was greater at 31°C than at 25°C, in both C. vulgaris and D. tertiolecta after 24 hours of exposure. These studies indicate that the experimental design (e.g. species tested, time point, concentration) influences the assessment of photosynthetic endpoints. In addition, these studies did not cross validate photosynthetic endpoints with later growth inhibition (Dewez and Oukarroum, 2012; Oukarroum et al., 2012; Navarro et al., 2015).
5.1.1. Light dependent photosynthesis and fluorescence

A simplified representation of the photosynthetic “Z scheme” is shown in Figure 5.1. This represents the series of light dependent photosynthetic reactions which generate adenosine triphosphate (ATP; through ATP synthase and the proton gradient generated across the thylakoid membrane by the electron transport chain and the oxygen evolving complex) and reduced nicotinamide adenine dinucleotide phosphate (NADPH; through the terminal reduction of NADP+ at ferredoxin-NADP+ reductase). Energy from photons of light are harvested by chlorophyll and accessory pigment-containing antenna complexes in photosystem I (PSI) and II (PSII). These antenna complexes direct this energy (in the form of excited electrons) to each photosystem’s respective reaction centre; Chlα protein complexes which excite electrons for use in the Z scheme (at a maximum light absorption spectrum of 700 and 680 nm for PSI and PSII respectively; Figure 5.1) (Jagannathan and Golbeck, 2009). Whilst photosynthetic processes such as the light independent Calvin cycle require the products from the light dependent reactions, detailed descriptions of these are outside the scope of this chapter and will not be further considered.
Figure 5.1: Representation of photosynthetic light reactions (aka the Z scheme), located on the thylakoid membrane of the chloroplast. Left to right: A photon (light) excites a single electron in the P680 reaction centre of photosystem II (PSII) to an elevated energy level, where it is passed to pheophytin (the primary electron acceptor in the electron transport chain). From here the electron is transferred to the primary (Qa), and then to the secondary plastoquinone acceptor (Qb), where it remains. Simultaneously, the oxidised P680 is reduced by the enzymatic photolysis of water (H2O) into oxygen (1/2O2), protons (2H+) and electrons (e-). An additional photon excites another single electron, which proceeds along the same pathway as the first to Qb. The now fully reduced QbH2 transfers the electron pair through cytochrome b6f complex and plastocyanin, where they act as the electron donors for the P700 reaction centre in photosystem I (PSI). From here the electrons are further excited by photons, and pass to a yet unidentified acceptor (analogous to pheophytin in PSII). The electrons pass through a series of iron sulphur proteins bound to the thylakoid membrane. The final electron acceptor is oxidised nicotinamide adenine dinucleotide phosphate (NADP+), where it is reduced to NADPH by ferredoxin-NADP+ reductase in the stroma of the chloroplast (adapted from Consalvey et al., 2005).

When light reaches the chloroplasts (containing the photosynthetic pigments) of plant/algal cells, the light-induced excitation of electrons in the pigments (including chlorophyll) of the light harvesting antenna complex is transferred through four different processes (Consalvey et al., 2005):

1. Non-specific transfer of energy to neighbouring molecules (non photochemical quenching; Table 5.1);
2. Emission of lower wavelength photon (red light) to that which excited it (fluorescence);
3. The transfer of energy to neighbouring chlorophyll molecules, and exciting their electrons;
4. The induction of a chemical reaction through the loss of the excited electron to the Z scheme (Figure 5.1).
These processes are inter-dependent, such that a relative increase in one will result in the decrease of another. The proportion of energy transferred down these four pathways can be altered by algal physiological state, which in turn is dependent on environmental conditions (Consalvey et al., 2005). By measuring fluorescence under certain conditions (Process 2 above), the efficiency of a variety of photochemical processes can be derived (Table 5.1).

In addition to fluorescence, photosynthetic processes can be assessed using oxygen evolution or radiolabelled carbon (¹⁴C) uptake (Consalvey et al., 2005). However such methods require specialised knowledge and training (setting up gas impermeable electrodes, handling of radio-isotopes, etc.), making fluorescence measurements more suitable for widespread use. To date, a number of photosynthetic toxicity endpoints (Table 5.1) have been investigated fluorimetrically in existing silver nanomaterial studies (Navarro et al., 2008; 2015; Miao et al., 2009; Dewez and Oukarroum, 2013; Oukarroum et al., 2012, Matorin et al., 2013), one of the most common is light adapted photosynthetic yield ($\Phi_{PSII}$). $\Phi_{PSII}$ of light adapted algal samples is assessed using a short pulse (a few hundred ms) of high intensity light (> 6,000 µmol/m²/s), and the fluorescence signal is measured both before (F) and immediately after the pulse is applied ($F_{m}'$). By subtracting the “steady state” F from the maximal $F_{m}'$, the variable fluorescence ($F_v'$) can be determined. Finally, by dividing $F_v'$ by $F_{m}'$, a ratio of variable to maximal fluorescence ($\Phi_{PSII}$) can be achieved, which is indicative of algal viability. This is based on the fact that in general the higher the level of plant stress, the lower the proportion of open reaction centres are available (and hence, the higher F). Upon the application of a high intensity light pulse, fluorescence is re-emitted proportional to the remaining open reaction centres (Ralph et al., 2007). Measuring $\Phi_{PSII}$ in dark adapted samples (i.e. a dark incubation of samples before reading – typically around 10 minutes), allows all photosystem II reaction centres to open, becoming fully oxidised and allowing dark adapted $F_{m}$ to be measured (Oukarroum et al., 2012; Dewez and Oukarroum, 2013). When performing $\Phi_{PSII}$ measurements on light adapted samples (i.e. reading samples directly from their incubation conditions), the resulting quantum yield is influenced more greatly by interactions between photosystems, the Calvin cycle, and the xanthophyll cycle (Demmig-Adams and Adams, 1996). Despite this, light-adapted $\Phi_{PSII}$ has been proposed as the preferred measure of toxicant stress due to its ecological validity – that is, environmental populations of algae would not be dark adapted (Ralph et al., 2007; Sjollema et al., 2014b). Any reference to $\Phi_{PSII}$ in this chapter should be assumed to be light adapted $\Phi_{PSII}$, unless otherwise stated. As $\Phi_{PSII}$ has a direct effect on growth rate, this endpoint was investigated for its potential to predict longer term endpoints such as 72 hour growth inhibition (Chapters 3 and 4).
Table 5.1: Common fluorimetrically-derived photosynthetic parameters (adapted from Consalvey et al., 2005 and Ralph et al., 2007)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Common abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum fluorescence yield</td>
<td>$F_0$ (dark) $F$ (light)</td>
<td>The baseline (dark) or steady state (light) fluorescence of chlorophyll. In dark adapted samples, all photosystem reaction centres are reduced. In light adapted samples, some photosystem reaction centres are reduced, and some oxidised. As fluorescence measured is the sum of all reaction centres $F_0$ is always $&lt; F$.</td>
</tr>
<tr>
<td>Maximum fluorescence yield</td>
<td>$F_{m}$ (dark) $F_{m'}$ (light)</td>
<td>The maximum re-emitted fluorescence after the application of a short duration/high intensity light (hundreds milliseconds and $&gt; 6,000 \mu$mol/m$^2$/s PPFD*). $F_{m'}$ is usually $&lt; F_m$ due to NPQ (see below) diverting a greater proportion of energy from the photosystem as heat in light adapted samples (and so there is less energy available to re-emit as fluorescence).</td>
</tr>
<tr>
<td>Variable fluorescence yield (Photochemical quenching)</td>
<td>$F_v$ (dark) $\Delta F, F_v, F_q$ (light)</td>
<td>The difference between maximum and minimum fluorescence yield in both dark adapted ($F_v$) and light adapted ($\Delta F, F_v, F_q$) samples. Light adapted variable fluorescence may also be known as quenched fluorescence, the proportion of light energy converted to chemical energy by the electron transport chain.</td>
</tr>
<tr>
<td>Maximum quantum yield</td>
<td>$F_v/F_m$, $[F_{m-F_0}]/F_m$</td>
<td>The maximum light utilisation efficiency, as measured in dark adapted samples.</td>
</tr>
<tr>
<td>Effective quantum yield</td>
<td>$\Phi_{PSII}$, $\Delta F/F_{m'}$, $[F_{m'-F}]/F_{m'}$, $F_v/F_{m'}$</td>
<td>The light utilisation efficiency at a given light level, in light adapted samples.</td>
</tr>
<tr>
<td>Non-photochemical quenching</td>
<td>$qN$, NPQ, $[F_{m'-F_{m}}]/F_{m'}$</td>
<td>The proportion of light energy dissipated as heat</td>
</tr>
<tr>
<td>Electron transport rate</td>
<td>ETR (absolute) rETR (relative)</td>
<td>The number of electrons transferred for a given amount of Chl$\alpha$ over time (µmol e$-$/µg Chl$\alpha$/s). This varies as a function of both light intensity (PPFD*, µmol/m$^2$/s) and light absorption coefficient of the plant/alga ($a$, m$^2$/µg Chl$\alpha$). As such, ETR is calculated by: $\Phi_{PSII} \times$ PPFD$*$ $\times a$ In cases where $a$ is not known, it can be omitted to give rETR.</td>
</tr>
</tbody>
</table>
Fast induction kinetics

OJIP curve

| Fast induction kinetics | OJIP curve | The change in Chla fluorescence in dark adapted samples < 1 s after a short duration/high intensity light pulse. The curve (fluorescence vs time) consists of four inflection points. Beginning at the origin (O, analogous to $F_0$), fluorescence initially quickly rises to point J, where it’s rise slows as it passes through I to the peak (P, analogous to $F_m$) |

*PPFD – Photon flux density. In electron transport rate calculations PPFD is sometimes divided by two to account for the distribution of light between photosystem I and II.

One advantage of photosynthetic inhibition tests is their potential to be performed over much shorter time periods. Short term (< 24 hour) photosynthetic efficiency inhibition has shown good agreement with 72 hour algal growth inhibition in a range of chemical toxicants (Masojidek et al., 2011). To date, existing studies have exposed algae to silver nanomaterials for periods ranging from hours (Navarro et al., 2008; 2015; Dewez and Oukarroum, 2012) to days (Miao et al., 2009; Oukarroum et al., 2012). For the work presented here, short term, high resolution photosynthetic inhibition tests were performed over 30 minutes. In addition, algae were also exposed to longer OECD 201 recommended exposures of 4 and 24 hours. The 4 hour timepoint was chosen to account for R. subcapitata’s slow silver uptake (Lee et al., 2004; 2005) and to allow NM300K suspensions time to reach equilibrium in the media (Navarro et al., 2008), and 24 hours was also chosen for direct comparison with the earliest timepoint in OECD 201 tests (Chapters 3 and 4).

Few studies of photosynthetic inhibition of silver nanomaterials have included ionic silver controls (Navarro et al., 2008; 2015). As discussed in Chapter 3, ionic controls are valuable when addressing the current debate of the contribution of ions to the toxicity of silver nanomaterials. Furthermore no studies have directly compared the photoinhibitory effect of silver (ionic or nanoparticulate) to toxicants of known modes of action. The photosynthetically active herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was commonly used in early studies of photosynthesis in algae (Metz et al., 1986; Ralph et al., 2007). DCMU selectively inhibits the electron transport chain in photosystem II by blocking the plastoquinone binding site (Figure 5.1) and effectively interrupting the photosynthetic process (Miles, 1995; Falkowski and Raven 2007). $K_2Cr_2O_7$ is recommended as a reference toxicant in some growth inhibition studies (OECD, 2011), and its mechanism of action in R. subcapitata is likely genotoxic (Labra et al., 2007). These two toxicants were chosen in order to provide a comparative effect to that of silver (both ionic and nanoparticulate) in R. subcapitata short term exposures (< 30 minutes) when assessing impacts on photosynthetic activity.
5.2. Aims and objectives

The aim of this chapter was to identify if assessment of photosynthetic activity in algae could support the development of more rapid tests to assess nanomaterial toxicity. This was achieved by addressing the following objectives:

- To investigate changes in *R. subcapitata* photosynthetic efficiency (ΦPSII) during short term exposure (30 minutes) to silver (both ionic and nanoparticle), DCMU and \( \text{K}_2\text{Cr}_2\text{O}_7 \) over a range of different toxicant concentrations.

- To identify if assessment of photosynthetic efficiency can be conducted in both standard (Chapter 3) and miniaturised (Chapter 4) OECD 201 test designs, at 4 and 24 hours.

- To assess the effect of ionic and nanoparticulate silver on *R. subcapitata* short term (4 and 24 hour) photosynthetic efficiency in the presence and absence of humic acid (0, 5 and 50 mg/l SRHA), and at a pH of 6 or 8.

- To compare results obtained from photosynthetic inhibition studies (concentration response, humic acid and pH studies) to growth inhibition data generated in Chapters 3 and 4.

5.3. Hypotheses

5.3.1. Research hypotheses

- Silver (\( \text{AgNO}_3 \) and NM300K), DCMU and \( \text{K}_2\text{Cr}_2\text{O}_7 \) stimulate a concentration dependent decrease in photosynthetic activity following exposure for 30 minutes.

- The four toxicants demonstrate a different pattern of toxicity due to their different mechanisms of action on *R. subcapitata* photosynthetic activity.

- Photosynthetic inhibition provides comparable data to previous chapters on growth inhibition tests (i.e. \( \text{EC}_{50} \); \( \text{AgNO}_3 \) toxicity > NM300K toxicity; time dependent reduction in toxicity) when assessed at 4 and 24 hours.

- In studies using humic acid and pH modified medium, 4 and 24 hour photosynthetic efficiency show the same patterns of toxicity for silver as described in previous chapters on growth inhibition (i.e. humic acid reduces
silver toxicity; pH 8 increases silver toxicity relative to pH 6/unbuffered medium).

5.3.2. Null hypotheses

- In 30 minute exposures silver (AgNO$_3$ and NM300K), DCMU and K$_2$Cr$_2$O$_7$ do not stimulate a concentration dependent decrease in photosynthetic activity.
- The four toxicants do not demonstrate a different pattern of toxicity on *R. subcapitata* photosynthetic activity.
- In 4 and 24 hour exposures, photosynthetic inhibition do not generate comparable data to previous chapters on growth inhibition tests.
- In studies using humic acid and pH modified medium, 4 and 24 hour photosynthetic efficiency do not show the same patterns of toxicity for silver as described in previous chapters on growth inhibition.

5.4. Methods

ΦPSII inhibition studies were performed using a PhytoPAM plankton analyser (Walz, Germany), and the associated PhytoWIN software package. The PhytoPAM analyser is a pulse amplitude modulation fluorimeter which measures chlorophyll fluorescence through excitation at multiple wavelengths using short pulses of light (µs), generated by an array of light emitting diodes. Samples were measured in 1.5-2 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 ($F_m'$) fluorescence were measured at 470, 645 and 665 nm and average ΦPSII calculated by taking an average of $F$ and $F_m'$ at the three wavelengths:

\[
ΦPSII = (F_m'-F)/F_m'
\]

5.4.1. 30 minute continuous exposures

Toxicant stocks of NM300K, AgNO$_3$ and K$_2$Cr$_2$O$_7$ were prepared as described in Section 2.4.1. and Section 3.4.1. DCMU stocks were prepared as described in Section 4.4.2. After preliminary range finding studies were performed, the following concentration ranges were chosen for NM300K (0, 100, 500, 1000 µg/l Ag), AgNO$_3$ (0, 10, 100, 500 µg/l Ag), K$_2$Cr$_2$O$_7$ (0, 0.1, 1, 10 mg/l), and DCMU (0, 2.6, 20, 100 µg/l). All toxicants were prepared in OECD medium.

Stock cultures of *R. subcapitata* were prepared as described in Section 3.4.2., and harvested for the experiment in the mid exponential growth phase. Final cell density in the
test cuvettes was $5 \times 10^4$ cells/ml, which was chosen in order to allow comparison with previous work presented in this thesis. Stock cultures were kept on an orbital shaker for the duration of the experiment (Multitron Standard, Infors-HT), at 225 RPM, under continuous fluorescent light \( \sim 120 \mu \text{mol/m}^2/\text{s} \), at 23 ±2°C). For each sample, 1 ml of algal stock culture was added to 500 µl of double strength OECD medium and 300 µl of 18 mΩ water. Each sample was placed into a PhytoPAM plankton analyser programmed to take ΦPSII measurements. Samples were read without toxicant for 5 minutes, before 200 µl of 18 mΩ water containing the desired toxicant concentration was added (or toxicant free for controls). Samples were then read every 20 seconds for 25 minutes. As the PhytoPAM can only process one sample at a time, all test concentrations were first analysed from highest to lowest toxicant concentration, before a new batch of replicate readings were performed. This was designed to avoid multiplication of errors due to potential drifts in stock culture (e.g. growth) over the duration of the experiment. Changes in stock culture cell density were measured using the \textit{in vivo} Chl\_a protocol described in Chapter 4.3.1d.

Thirty minute photosynthetic exposures (and previous range finding experiments) were performed by Elise Joonas (University of Tartu, Estonia), under the supervision of the author during a short term Erasmus internship at Heriot Watt University. Experimental design, data analysis, and interpretation were carried out by the author.

5.4.2. ΦPSII concentration response tests

ΦPSII inhibition tests were performed using both standard (Chapter 3) and miniaturised (Chapter 4) OECD 201 tests in OECD medium. From each test system, 1.5 ml of each sample was removed and placed in a quartz cuvette. Furthermore, the ΦPSII of each sample was measured using a PhytoPAM plankton analyser after 4 and 24 hours of exposure.

ΦPSII inhibition was calculated for each individual treatment replicate, relative to the mean growth rate of the control, using the following equation (based on growth inhibition calculations in Section 3.4.3):

\[
\%I_{\Phi\text{PSII}} = \left(\frac{\Phi\text{PSII}_{\text{control}} - \Phi\text{PSII}_{\text{treatment}}}{\Phi\text{PSII}_{\text{control}}}\right) \times 100
\]

Where: \(\%I_{\Phi\text{PSII}}\) = percent ΦPSII inhibition; \(\Phi\text{PSII}_{\text{control}}\) = average ΦPSII in the control group; \(\Phi\text{PSII}_{\text{treatment}}\) = average ΦPSII in the treatment replicate.

Photosynthetic inhibition tests were also performed (after 4 and 24 hours) on pH and humic acid studies in OECD medium, as described in Chapter 3 (Flasks) and Chapter 4 (24 well plates).
5.4.3. Statistical analysis

Details of statistical testing software and procedures can be found in Section 2.4.5. In 30 minute continuous exposures of each toxicant, \textit{R. subcapitata} ΦPSII was compared across toxicant concentrations to equivalent toxicant free controls at the end of the test, to determine which concentrations induced a significantly different change in ΦPSII.

In concentration response and humic/pH studies, \textit{R. subcapitata} control ΦPSII was compared between each timepoint (4 and 24 hours). For concentration response data a probit model was fitted to ΦPSII inhibition data generated at each timepoint. EC$_{50}$ (µg/l) was compared between timepoints, for DCMU, NM300K, AgNO$_3$.

\textit{R. subcapitata} ΦPSII in toxicant free humic acid concentrations (0, 5 and 50 mg/l SRHA) and pH levels (unadjusted, pH 6 and pH 8) were compared at each timepoint. The effect of humic acid/pH on ΦPSII inhibition were analysed at each timepoint, and compared to their appropriate toxicant free controls.

5.5. Results

5.5.1. 30 minute continuous exposures

ΦPSII in control cultures was between 0.65 and 0.7 in all experiments, and remained at this level for the duration of the 30 minute experiments (Figures 5.2-5.5). After 5 minutes, the addition of DCMU, AgNO$_3$ and NM300K all induced an immediate and continuous decline in ΦPSII, over the duration of the test, relative to control cultures (Figures 5.2, 5.4 and 5.5). In DCMU, this response was clearly concentration-dependent with a decrease in ΦPSII with increasing concentration (Figure 5.2). After 30 minutes ΦPSII in 20 and 100 µg/l DCMU exposures were significantly lower than toxicant free controls (F = 348.47, p < 0.001), with a relative inhibition of 3.62 (±0.43), 18.4 (±2.5) and 65.76 (±1.95) % for 2.6, 20 and 100 µg/l DCMU respectively. By contrast, K$_2$Cr$_2$O$_7$ concentration had no effect on ΦPSII, which showed no significant difference in \textit{R. subcapitata} ΦPSII relative to control cultures over the 30 minute test duration at concentrations < 100 mg/l (Figures 5.3).
Figure 5.2: Impact of DCMU [0-100 µg/l] on *R. subcapitata* photosynthetic efficiency (ΦPSII) over 30 minutes of exposure in OECD medium. Toxicant was added after a 5 minute acclimation period and effect on ΦPSII was measured for the remaining 25 minutes. Data are expressed as mean ΦPSII and standard error of the mean (n = 1, one experiment with three replicates).
AgNO$_3$ and NM300K stimulated a concentration dependent effect on photosynthetic activity (Figures 5.4 and 5.5). However unlike DCMU experiments, silver induced ΦPSII inhibition appeared to reach a plateau, where higher concentrations did not result in further inhibition of photosynthesis. For AgNO$_3$, a significant difference in 30 minute ΦPSII inhibition was observed between concentrations ($F = 50.38$, $p < 0.001$), however concentrations > 100 µg/l Ag showed no further decrease in 30 minute ΦPSII (Figure 5.4). At 30 minutes, ΦPSII inhibition for AgNO$_3$ was 7.99 (±1.65), 33.36 (±1.85) and 27.02 (±1.95) % for 10, 100 and 500 µg/l Ag respectively. Comparing these results to NM300K, a similar significant difference was observed between silver concentrations ($F = 12.59$, $p = 0.002$), with concentrations > 500 µg/l Ag showing no further reduction in 30 minute ΦPSII (Figure 5.5). The 30 minute ΦPSII inhibition for NM300K was 8.52 (±4.96), 36.99 (±3.94) and 40.73 (±8.95) for 10, 500 and 1000 µg/l Ag respectively. In initial range finding experiments, silver concentrations (both ionic and nanoparticulate) above the concentrations described here induced no further effect on $R$. subcapitata ΦPSII (results not shown). Whilst these higher silver concentrations (100 and 500 µg/l Ag for AgNO$_3$; 500 and 1000 µg/l Ag for NM300K) induced a significantly lower 30 minute ΦPSII relative to controls (~30% and ~39% for AgNO$_3$ and NM300K respectively), the effect was less than observed for the highest concentration of DCMU (Figures 5.2 cf. Figures 5.4 and 5.5).
Figure 5.4: Impact of AgNO₃ [0-500 µg/l Ag] on *R. subcapitata* photosynthetic efficiency (ΦPSII) over 30 minutes of exposure in OECD medium. Toxicant was added after a 5 minute acclimation period and effect on ΦPSII was measured for the remaining 25. Data expressed as mean ΦPSII and standard error of the mean (n = 1, one experiment with three replicates).
Figure 5.5: Impact of NM300K [0-1000 µg/l Ag] on R. subcapitata photosynthetic efficiency (ΦPSII) over 30 minutes of exposure in OECD medium. Toxicant was added after a 5 minute acclimation period and effect on ΦPSII was measured for the remaining 25. Data expressed as mean ΦPSII and standard error of the mean (n = 1, one experiment with three replicates).

5.5.2. ΦPSII concentration response tests

Results described here are based on the standard OECD test design (250 ml flasks). A high degree of similarity was observed in tests performed in 24 well plates and so these results are presented separately in Appendix 6 to avoid repetition. Control culture (toxicant free) ΦPSII was 0.65-0.7 at 4 and 24 hours, in both flasks and well plates. Toxicity data generated using 4 and 24 h ΦPSII inhibition as an endpoint were similar to the standard 72 hour growth inhibition endpoint, in both flasks (Chapter 3) and well plates (Chapter 4) (Table 5.5).

DCMU (Figure 5.6, Table 5.2), AgNO₃ (Figure 5.7, Table 5.3) and NM300K (Figure 5.8, Table 5.4) showed a concentration dependent impact on R. subcapitata ΦPSII inhibition. As concentrations of DCMU increased, so did ΦPSII inhibition. At the lowest DCMU concentration (0.2 µg/l) ΦPSII inhibition was 0.16 (±0.99) and 0.75 (±0.27) % after 4 and 24 hours of exposure respectively (Figure 5.6). At the highest DCMU concentration tested (100 µg/l) ΦPSII inhibition was 91.03 (±0.64) and 95.98 (±6.33) % after 4 and 24 hours of exposure respectively (Figure 5.6). On the basis of ΦPSII, no significant difference in toxicity was observed between timepoints, with an EC₅₀ value of 28.15 (±1.29) and 22.89 (±1.05) µg/l at 4 and 24 hours, respectively (Figure 5.6, Table 5.2).
For AgNO₃, the lowest concentration tested (0.2 µg/l Ag) induced a slight (but not significant) hormetic effect on ΦPSII at both timepoints, with a ΦPSII inhibition of -0.75 (±0.46) and -0.48 (±0.58) % after 4 and 24 hours of exposure respectively (Figure 5.7). At concentrations greater than 0.6 µg/l there was a concentration dependent increase in ΦPSII inhibition. At the highest concentration tested (20 µg/l Ag) ΦPSII inhibition was 97.38 (±0.39) and 78.11 (13.62) % after 4 and 24 hours of exposure respectively (Figure 5.7). AgNO₃ induced ΦPSII toxicity was significantly higher (T = -7.38, p = 0.018) at 4 hours than at 24 hours, with an EC₅₀ value of 4.12 (±0.17) and 11.48 (±0.39) µg/l at 4 and 24 hours respectively (Figure 5.7, Table 5.3).

For NM300K, the lowest concentration tested (30 µg/l Ag) induced a ΦPSII inhibition of 27.73 (±9.73) and 2.69 (±0.92) % after 4 and 24 hours of exposure respectively (Figure 5.8). At the highest NM300K concentration (150 µg/l Ag) ΦPSII inhibition was 64.52 (±6.52) and 38.02 (9.36) % after 4 and 24 hours of exposure respectively (Figure 5.8). Whilst NM300K induced a 50 % reduction in R. subcapitata ΦPSII at concentrations of 106.74 (±2.57) and 164.51 (±3.46) µg/l Ag at 4 and 24 hours respectively, no significant difference was observed between these timepoints (Figure 5.8, Table 5.4). Compared to NM300K, AgNO₃ was between 14-26 times more toxic.
**Figure 5.6**: Impact of DCMU [0.2-100 µg/l] on *R. subcapitata* photosynthetic efficiency (ΦPSII) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates).

**Table 5.2**: Probit modelled effective concentrations of DCMU [0.2-100 µg/l] on *R. subcapitata* photosynthetic efficiency (ΦPSII; 4 and 24 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model of DCMU induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 1, one experiment with three replicates).

<table>
<thead>
<tr>
<th>DCMU (µg/l)</th>
<th>4 hour (µg/l) ± SE</th>
<th>24 hour (µg/l) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Not achieved</td>
<td>Not achieved</td>
</tr>
<tr>
<td>20</td>
<td>0.46 (±1.7)</td>
<td>1.57 (±1.34)</td>
</tr>
<tr>
<td>50</td>
<td>28.15 (±1.29)</td>
<td>22.89 (±1.05)</td>
</tr>
<tr>
<td>80</td>
<td>55.84 (±1.91)</td>
<td>44.21 (±1.55)</td>
</tr>
</tbody>
</table>
Figure 5.7: Impact of AgNO₃ [0.2-20 µg/l Ag] on *R. subcapitata* photosynthetic efficiency (ΦPSII) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates).

Table 5.3: Probit modelled effective concentrations of AgNO₃ [0.2-20 µg/l Ag] on *R. subcapitata* photosynthetic efficiency (ΦPSII; 4 and 24 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model of AgNO₃ induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 1, one experiment with three replicates).

<table>
<thead>
<tr>
<th>AgNO₃ (µg/l Ag)</th>
<th>EC₅₀ (%)</th>
<th>4 hour</th>
<th>24 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Not achieved</td>
<td>1.14 (±0.42)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.99 (±0.18)</td>
<td>4.69 (±0.35)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4.12 (±0.17)</td>
<td>11.48 (±0.39)</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>7.25 (±0.28)</td>
<td>18.27 (±0.6)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.8: Impact of NM300K [30-150 µg/l Ag] on R. subcapitata photosynthetic efficiency (ΦPSII) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean (n = 3).

Table 5.4: Probit modelled effective concentrations of NM300K [30-150 µg/l Ag] on R. subcapitata photosynthetic efficiency (ΦPSII; 4 and 24 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model of NM300K induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 3).

<table>
<thead>
<tr>
<th>NM300K (µg/l Ag)</th>
<th>EC₅₀ (%)</th>
<th>4 hour</th>
<th>24 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Not achieved</td>
<td></td>
<td>55.75 (±2.94)</td>
</tr>
<tr>
<td>20</td>
<td>Not achieved</td>
<td></td>
<td>93.08 (±1.93)</td>
</tr>
<tr>
<td>50</td>
<td>106.74 (±2.57)</td>
<td>&gt; 150</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>&gt; 150</td>
<td></td>
<td>&gt; 150</td>
</tr>
</tbody>
</table>

A comparison of toxicity data (EC₅₀ value) between the different test designs was presented in Table 5.5. Photosynthetic (ΦPSII) inhibition (4 and 24 hours) results were presented in detail in this chapter, whilst growth inhibition (24, 48, and 72 hours) results were presented in Chapter 3 (AgNO₃ and NM300K, using in vitro Chlₐ) and Chapter 4 (DCMU, AgNO₃)
and NM300K, using *in vivo* Chl$_a$). Full concentration response data for DCMU using *in vitro* Chl$_a$ were presented in Appendix 4, and ΦPSII inhibition (AgNO$_3$ and NM300K, 4 and 24 hours) data using the 24 well plate design were presented in Appendix 6. Despite differences in absolute toxicity (DCMU, AgNO$_3$ and NM300K) between test systems/endpoints, similar patterns of toxicity were observed. Namely, the toxicity of all three substances followed the order AgNO$_3$ > DCMU > NM300K. In addition, the toxicity of both forms of silver decreased over time (higher EC$_{50}$ value), whereas DCMU toxicity was comparatively more stable.

**Table 5.5**: Probit modelled of short term (4 and 24 hours) photosynthetic inhibition (ΦPSII) EC$_{50}$ values, compared to longer term (24, 48, and 72 hours) growth inhibition EC$_{50}$ values (measured using both *in vivo* and *in vitro* Chl$_a$ methods; see chapters 3 and 4) in *R. subcapitata* in OECD medium. EC$_{50}$ expressed as µg/l DCMU for DCMU, and µg/l Ag for both AgNO$_3$ and NM300K. Standard error of the mean in parentheses.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Toxicant (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCMU</td>
</tr>
<tr>
<td>Photosynthetic inhibition</td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>28.15 (±1.29)</td>
</tr>
<tr>
<td>24 h</td>
<td>22.89 (±1.05)</td>
</tr>
<tr>
<td>Growth inhibition</td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> Chl$_a$</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>63.76 (±2.01)</td>
</tr>
<tr>
<td>48 h</td>
<td>47.42 (±1.49)</td>
</tr>
<tr>
<td>72 h</td>
<td>43.20 (±1.36)</td>
</tr>
<tr>
<td><em>In vivo</em> Chl$_a$</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>31.54 (±1.09)</td>
</tr>
<tr>
<td>48 h</td>
<td>25.17 (±0.79)</td>
</tr>
<tr>
<td>72 h</td>
<td>27.08 (±0.82)</td>
</tr>
</tbody>
</table>

**5.5.3. Impact of humic acid on silver induced photosynthesis inhibition**

The addition of SRHA had a small but significant effect on ΦPSII in toxicant free controls. In control treatments without humic acid or toxicant, *R. subcapitata* ΦPSII was 0.65 (±0.001) and 0.68 (±0.001), at 4 and 24 hours respectively. A significant increase in 4 hour ΦPSII was observed relative to humic free controls, for both humic acid concentrations (H = 6.58, p = 0.014), with a ΦPSII of 0.66 (±0.01) and 0.67 (±0.01) for 5 and 50 mg/l SRHA respectively. At 24 hours humic acid also had a significant effect on ΦPSII (H = 16.77, p < 0.001), however 5 mg/l SRHA resulted in a significantly lower ΦPSII (0.67 ±0.008) relative to
the control, whereas in cultures containing 50 mg/l SRHA it was significantly higher (0.69 ±0.003).

The toxicity of NM300K [190 µg/l] was reduced in the presence of humic acid (Figure 5.9). At 4 hours, a significant difference in NM300K mediated ΦPSII inhibition was observed in the presence of SRHA (H = 23.14, p < 0.001) with a ΦPSII inhibition of 87.58 (±1.01), 82.24 (±1.18) and 1.07 (±2.67) % for 0, 5 and 50 mg/l SRHA respectively, indicating a SRHA concentration dependent effect. A similar finding was obtained at 24 hours (F = 188.88, p < 0.001), with a NM300K induced ΦPSII inhibition of 68.35 (±3.05), 18.73 (±2.42) and 4.48 (±1.63) % for 0, 5 and 50 mg/l SRHA respectively. NM300K mediated ΦPSII inhibition decreased significantly over time for both humic acid free (T = 5.99, p < 0.001) and 5 mg/l SRHA (T = 23.58, p < 0.001) test conditions. By contrast, in OECD medium containing 50 mg/l SRHA, no significant difference in NM300K mediated ΦPSII inhibition was observed between the timepoints studied.

**Figure 5.9:** Impact of NM300K [190 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage of control and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (4 hours, Kruskal-Wallis; 24 hours, one way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.
The toxicity of AgNO₃ [6 µg/l] was reduced in the presence of SRHA (Figure 5.10). At 4 hours, ΦPSII inhibition was significantly different between SRHA conditions (F = 5058.89, p < 0.001), resulting in 76.96 (±0.38), 73.26 (±0.13) and 2.59 (±0.94) % inhibition for 0, 5 and 50 mg/l SRHA respectively, indicating a SRHA concentration dependent effect. A similar significant difference was observed at 24 hours (F = 18.67, p = 0.003), with AgNO₃ induced ΦPSII inhibition of 33.17 (±5.28), 19.75 (±0.62) and 6.24 (±0.96) % for 0, 5 and 50 mg/l SRHA respectively. Similarly to AgNO₃ concentration-response studies (Figure 5.6, Table 5.2), AgNO₃ induced ΦPSII inhibition decreased significantly over time for both humic acid free (T = 8.28, p = 0.014) and 5 mg/l SRHA (T = 83.81, p < 0.001) test conditions. However in OECD medium containing 50 mg/l SRHA, no significant difference in AgNO₃ induced ΦPSII inhibition was observed over time (4 and 24 hours).

**Figure 5.10**: Impact of AgNO₃ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. AgNO₃ photosynthetic inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.
5.5.4. Impact of pH on silver induced photosynthesis inhibition

No significant differences in NM300K [190 µg/l] mediated ΦPSII inhibition was observed at pH 6 or 8, at both 4 and 24 hours (Figure 5.11). At 4 hours, NM300K induced ΦPSII inhibition was 76.39 (±5.17), 72.98 (±7.97) and 90.09 (±2.13) % in unbuffered OECD medium, and medium buffered to pH 6 and pH 8 respectively. By comparison, at 24 hours, NM300K induced ΦPSII inhibition was 59.11 (±9.50), 66.52 (±12.90) and 88.03 (±4.30) % in unbuffered OECD medium, and medium buffered to pH 6 and pH 8 respectively. Although NM300K toxicity decreased over time in all three pH conditions, this effect was not found to be significant.

Figure 5.11: Impact of NM300K [190 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different.

AgNO₃ [6 µg/l] mediated ΦPSII inhibition was significantly higher at pH 8 than both unbuffered medium and medium buffered to pH 6 (F = 66.60, p <0.001), with a ΦPSII inhibition of 86.23 (±0.20), 69.10 (±0.30) and 69.73 (±2.03) % respectively at 4 hours, (Figure 5.12). A similar significant difference was observed at 24 hours (F = 54.31, p <0.001), with an AgNO₃ induced ΦPSII inhibition of 27.20 (±1.29), 53.41 (±3.74) and 74.71 (±3.95) % in unbuffered
OECD medium, and medium buffered to pH 6 and pH 8 respectively. Similarly to AgNO₃ concentration-response studies (Figure 5.6, Table 5.2), AgNO₃ induced ΦPSII inhibition decreased significantly over time for both unbuffered medium (T = 31.61, p < 0.001) and medium buffered to pH 6 (T = 3.84, p = 0.031). However in OECD medium buffered to pH 8, timepoint showed no significant difference in AgNO₃ induced ΦPSII inhibition.

Figure 5.12: Impact of AgNO₃ [6 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on R. subcapitata photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

5.5.5. Short term ΦPSII toxicity tests as a predictor of silver toxicity using the standard 72 hour growth inhibition endpoint

Finally, the findings from photosynthetic efficiency experiments from all humic acid and pH studies were compared with the findings obtained from the 72 hour OECD growth rate studies (Section 3.5.1.). A summary of the effect of NM300K [190 µg/l] and AgNO₃ [6 µg/l] on both photosynthetic efficiency and growth rate in R. subcapitata was presented in Table 5.6 for humic acid studies, and Table 5.7 for pH studies. Similar patterns of toxicity were observed using either endpoint, these were:
1. AgNO₃ was more toxic than NM300K on a silver mass basis (Tables 5.6 and 5.7).

2. Both forms of silver showed a reduced toxicity as exposure time increases, for both photosynthetic inhibition (4 and 24 hours) and growth inhibition (24, 48 and 72 hours) (Tables 5.6 and 5.7).

3. Increasing SRHA concentration reduces silver (ionic and particulate) toxicity in a concentration dependent manner (Table 5.6).

4. Silver toxicity in OECD medium buffered to pH 8 was generally higher than either unbuffered medium or medium buffered to pH 6 (Table 5.7).

Despite these similarities, the principal difference between the two endpoints was that photosynthetic inhibition never exceeded 100 %, whereas growth inhibition often did, especially at earlier timepoints. This was related to the endpoints/measurement methods themselves; as photosynthetic efficiency was only measured in viable cells (containing Chl a pigments) it is not possible to record > 100 % inhibition. By contrast, in growth inhibition studies an inhibition of > 100 % indicates cell death, as the cell density decreases to less than the initial inoculum density.
Table 5.6: The impact of AgNO$_3$ [6 µg/l] and NM300K [190 µg/l] on short term (4 and 24 hours) photosynthetic inhibition (ΦPSII), compared to longer term (24, 48, and 72 hours) growth inhibition (measured using both *in vivo* and *in vitro* Chl$_a$ methods; see chapters 3 and 4) in *R. subcapitata* in OECD medium with different levels of Suwannee River humic acid (0, 5 and 50 mg/l). Standard error of the mean in parentheses.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Inhibition (%)</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NM300K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photosynthetic inhibition</td>
<td>190 µg/l</td>
<td>190 µg/l + 5 mg/l SRHA</td>
<td>190 µg/l + 50 mg/l SRHA</td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>87.58 (±1.01)</td>
<td>82.58 (±1.18)</td>
<td>1.07 (±2.68)</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>68.35 (±3.05)</td>
<td>18.73 (±2.42)</td>
<td>4.48 (±1.63)</td>
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</tr>
<tr>
<td>Growth inhibition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> Chl$_a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>202.03 (±6.62)</td>
<td>156.66 (±4.04)</td>
<td>31.98 (±4.17)</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>153.66 (±7.76)</td>
<td>117.02 (±5.04)</td>
<td>46.00 (±4.59)</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>111.63 (±5.56)</td>
<td>96.07 (±5.48)</td>
<td>48.12 (±8.24)</td>
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<tr>
<td><em>In vivo</em> Chl$_a$</td>
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<td></td>
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<td></td>
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<tr>
<td>24 h</td>
<td>240.80 (±17.90)</td>
<td>201.03 (±11.76)</td>
<td>39.81 (±5.08)</td>
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<td>48 h</td>
<td>169.38 (±2.02)</td>
<td>126.29 (±4.05)</td>
<td>40.86 (±1.75)</td>
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</tr>
<tr>
<td>72 h</td>
<td>133.08 (±5.63)</td>
<td>102.41 (±3.09)</td>
<td>42.14 (±5.14)</td>
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</tr>
<tr>
<td>AgNO$_3$</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Photosynthetic inhibition</td>
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<td>6 µg/l + 5 mg/l SRHA</td>
<td>6 µg/l + 50 mg/l SRHA</td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>76.96 (±0.38)</td>
<td>73.26 (±0.13)</td>
<td>2.59 (±0.94)</td>
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<tr>
<td>24 h</td>
<td>33.17 (±5.28)</td>
<td>19.75 (±0.62)</td>
<td>6.24 (±0.96)</td>
<td></td>
</tr>
<tr>
<td>Growth inhibition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> Chl$_a$</td>
<td></td>
<td></td>
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<tr>
<td>24 h</td>
<td>138.89 (±6.60)</td>
<td>119.81 (±10.45)</td>
<td>40.67 (±0.95)</td>
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<td>91.66 (±2.07)</td>
<td>41.78 (±1.90)</td>
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<td>72 h</td>
<td>39.76 (±7.45)</td>
<td>55.36 (±3.85)</td>
<td>25.34 (±2.83)</td>
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</tr>
<tr>
<td><em>In vivo</em> Chl$_a$</td>
<td></td>
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<td></td>
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<tr>
<td>24 h</td>
<td>185.84 (±2.92)</td>
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<tr>
<td>48 h</td>
<td>108.62 (±7.62)</td>
<td>95.28 (±1.77)</td>
<td>33.32 (±0.59)</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>63.21 (±8.82)</td>
<td>65.22 (±7.01)</td>
<td>31.18 (±3.08)</td>
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</table>
Table 5.7: The impact of AgNO₃ [6 µg/l] and NM300K [190 µg/l] on short term (4 and 24 hours) photosynthetic inhibition (ΦPSII), compared to longer term (24, 48, and 72 hours) growth inhibition (measured using both in vivo and in vitro Chl a methods; see chapters 3 and 4) in R. subcapitata in OECD medium with different levels of pH modification (unbuffered, pH 6 and pH 8). Standard error of the mean in parentheses.

<table>
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<tr>
<th>Endpoint</th>
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<td></td>
<td>190 µg/l pH 6</td>
<td>190 µg/l pH 8</td>
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<tr>
<td>Photosynthetic efficiency</td>
<td>190 µg/l unbuffered</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>76.39 (±5.17)</td>
<td>72.98 (±7.97)</td>
<td>90.09 (±2.13)</td>
</tr>
<tr>
<td>24 h</td>
<td>59.11 (±9.50)</td>
<td>66.52 (±12.90)</td>
<td>88.03 (±4.30)</td>
</tr>
<tr>
<td>Growth inhibition</td>
<td></td>
<td></td>
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<tr>
<td>In vitro Chl a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>147.97 (±13.42)</td>
<td>136.11 (±22.71)</td>
<td>267.80 (±17.73)</td>
</tr>
<tr>
<td>48 h</td>
<td>78.39 (±12.13)</td>
<td>117.45 (±23.87)</td>
<td>165.36 (±13.61)</td>
</tr>
<tr>
<td>72 h</td>
<td>59.80 (±11.17)</td>
<td>84.69 (±21.15)</td>
<td>123.41 (±13.15)</td>
</tr>
<tr>
<td>In vivo Chl a</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>202.40 (±18.77)</td>
<td>149.22 (±17.97)</td>
<td>264.63 (±12.32)</td>
</tr>
<tr>
<td>48 h</td>
<td>128.71 (±12.69)</td>
<td>122.17 (±15.58)</td>
<td>168.95 (±5.79)</td>
</tr>
<tr>
<td>72 h</td>
<td>82.11 (±10.85)</td>
<td>90.36 (±16.75)</td>
<td>131.97 (±8.26)</td>
</tr>
<tr>
<td>AgNO₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photosynthetic efficiency</td>
<td>6 µg/l unbuffered</td>
<td>6 µg/l pH 6</td>
<td>6 µg/l pH 8</td>
</tr>
<tr>
<td>4 h</td>
<td>69.10 (±0.30)</td>
<td>69.73 (±2.03)</td>
<td>86.23 (±0.20)</td>
</tr>
<tr>
<td>24 h</td>
<td>27.20 (±1.29)</td>
<td>53.41 (±3.74)</td>
<td>74.71 (±3.95)</td>
</tr>
<tr>
<td>Growth inhibition</td>
<td></td>
<td></td>
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<tr>
<td>In vitro Chl a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>116.61 (±6.65)</td>
<td>100.28 (±1.78)</td>
<td>258.25 (±53.87)</td>
</tr>
<tr>
<td>48 h</td>
<td>72.45 (±3.31)</td>
<td>87.10 (±1.77)</td>
<td>139.89 (±6.20)</td>
</tr>
<tr>
<td>72 h</td>
<td>34.59 (±4.97)</td>
<td>49.56 (±2.95)</td>
<td>94.10 (±5.79)</td>
</tr>
<tr>
<td>In vivo Chl a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>169.03 (±2.44)</td>
<td>151.62 (±2.26)</td>
<td>220.39 (±32.82)</td>
</tr>
<tr>
<td>48 h</td>
<td>80.30 (±3.48)</td>
<td>100.10 (±1.31)</td>
<td>150.12 (±5.67)</td>
</tr>
<tr>
<td>72 h</td>
<td>42.57 (±4.99)</td>
<td>60.31 (±3.30)</td>
<td>101.65 (±4.60)</td>
</tr>
</tbody>
</table>

Finally, a significant linear relationship was found between 72 hour growth rate and both 4 ($r^2 = 0.64, p < 0.001$) and 24 hour ($r^2 = 0.72, p < 0.001$) ΦPSII inhibition (Figure 5.13). Accordingly, short term photosynthetic efficiency tests (ΦPSII) showed a positive relationship with the standard OECD 201 72 hour growth inhibition endpoint. For ΦPSII, the timepoint...
assessed was important, with 24 hour ΦPSII showing a stronger relationship \( r^2 = 0.72 \) with 72 hour growth inhibition than 4 hour ΦPSII \( r^2 = 0.64 \). This indicated that measurement of ΦPSII at 24 hours was more predictive of resultant growth rate in the standard OECD 201 test. Interestingly, sources of variation for 4 and 24 hour ΦPSII, compared to 72 hour growth rate emerged in different ways. For 4 hour ΦPSII, a sample with low photosynthetic efficiency (< 0.4) was more likely to show a 72 hour growth rate of > 1 d\(^{-1}\). Conversely, for 24 hour ΦPSII, samples with high photosynthetic efficiency (> 0.4) were more likely to result in a 72 hour growth rate of < 1 d\(^{-1}\). This means that 4 hour ΦPSII may underestimate 72 hour growth rate, whereas 24 hour ΦPSII may overestimate it.

**Figure 5.13:** Comparison of data obtained from OECD 201 algal growth inhibition testing and photosynthetic efficiency for both NM300K and AgNO\(_3\) toxicity to *R. subcapitata* in OECD medium, at 4 (black circles, solid line) and 24 (grey squares, dashed line) hours. Cells were exposed to Suwannee River humic acid (0, 5 and 50 mg/l) and pH modification (unbuffered, pH 6 and pH 8 using 3.5 mM 3-(N-morpholino)propanesulfonic acid), containing either no toxicant, AgNO\(_3\) [6 µg/l Ag] or NM300K [190 µg/l Ag]. A positive correlation was observed between 72 hour *in vivo* growth rate and photosynthetic efficiency at 4 \( (r^2 = 0.64; p < 0.001) \) and 24 \( (r^2 = 0.72; p < 0.001) \) hours \( n = 168 \).
5.6. Discussion

The suitability of short term (< 24 hours) ΦPSII (photosynthetic efficiency) tests to assess the algal toxicity of nanomaterials as an alternative to the OECD 201 72 hour growth inhibition test was investigated. Of benefit, is that use of this alternative method can also provide information regarding the mechanism of toxicity.

5.6.1. 30 minute ΦPSII inhibition test

The toxicity of NM300K, AgNO₃, DCMU and K₂Cr₂O₇ were assessed in a 30 minute photosynthetic efficiency test. The hypothesis that all four toxicants would stimulate concentration dependent decrease in ΦPSII was accepted for all toxicants except K₂Cr₂O₇, which showed no effect at any of the concentrations studied. DCMU was the most toxic, and demonstrated a clear concentration dependent effect on ΦPSII. In studies with NM300K and AgNO₃, ΦPSII inhibition reached a maximum level, where higher concentrations had no further effect.

Of interest was that both reference toxicants (DCMU and K₂Cr₂O₇) behaved very differently over the 30 minute ΦPSII inhibition studies. For DCMU, 30 minute test concentrations were chosen to be comparable with 72 hour growth inhibition concentration response data presented in Chapter 3. However for K₂Cr₂O₇, pilot studies revealed that test concentrations used in growth inhibition studies (Appendix 3), were insufficient to generate a response in 30 minute ΦPSII studies. As a result a wide concentration range was chosen in these studies in an attempt to elicit a toxic effect. Despite this, K₂Cr₂O₇ failed to elicit a change in *R. subcapitata* ΦPSII over the 30 minute exposure, whereas DCMU had a clear concentration dependent effect. This observation is highly likely to be indicative of the different mechanism of action in the two toxicants. DCMU has a specific effect on photosynthesis by blocking the electron transport chain at plastoquinone, effectively disrupting photosynthesis (Miles, 1995; Falkowski and Raven, 2007). K₂Cr₂O₇ is thought to be genotoxic, inducing mutations throughout the genome of *R. subcapitata* (Labra et al., 2007). Vannini *et al.* (2009) found that K₂Cr₂O₇ targets gene expression and disrupts the production of a number of photosynthetic proteins, including RuBisCO, light harvesting Chl₅/Chl₆ complex, and Light-Harvesting Complex Stress-Related (LHCSR) proteins. However as such photosynthetic effects derive from changes gene expression which require multiple generations to be expressed, this may likely explain the lack of effect over the 30 minute exposure used here. In addition, this may also explain why K₂Cr₂O₇ has a lower EC₅₀ over the 72 hour OECD 201 test, which is specifically designed to observe toxic effects on multiple generations of algae (OECD, 2011). Accordingly, the use of the ΦPSII test presented here may potentially provide insight into the mechanism of toxicity of
chemicals, but may be too short in duration to be suitable for generating concentration-response data which can be generalised to standard test endpoints (i.e. OECD 201 growth inhibition).

Whilst toxicants differed in their effect on 30 minute ΦPSII inhibition, by comparing the response of \textit{R. subcapitata} ΦPSII to both ionic silver and nanoparticles to the reference toxicants. As observed for DCMU, a rapid decline in ΦPSII was observed following exposure of algae to both forms of silver. However for both AgNO₃ and NM300K the decline in ΦPSII was smaller in magnitude compared to DCMU. Furthermore, the effect of both silver ions and nanoparticles on ΦPSII reached saturation, where an increase in toxicant concentration did not result in greater ΦPSII inhibition. As discussed in Chapter 3, \textit{R. subcapitata} is considered to exhibit slow silver uptake from its surrounding medium (Lee et al., 2004; 2005). This may explain the saturation effect observed here, as silver uptake is thought to be mediated through cationic Cu(I) transporters, the uptake rate of silver over short exposures will naturally be limited by the total number of such transporters at the cell surface (Lee et al., 2004). Future work may investigate this saturation effect by comparing these results with species exhibiting faster silver uptake rates, such as \textit{Chlamydomonas reinhardtii} (Lee et al., 2005; Navarro et al., 2008; Dewez and Oukarroum et al., 2012). Alternatively, the addition of compounds known to increase silver uptake, such as thiosulphate and other sulphurous ligands (Fortin and Campbell, 2001; Kramer et al., 2002; Hiriart-Baer et al., 2006), could be used in combination with silver to determine whether such compounds further reduce short term ΦPSII.

These results show that both ionic silver and nanoparticles exerted a rapid effect on reducing ΦPSII in \textit{R. subcapitata}. By measuring ΦPSII, this suggests that photosystem II may be one of the target sites for silver’s toxic effect, by reducing the efficiency of electron transfer between photosystem II and plastoquinone (Ralph et al., 2007; Dewez and Oukarroum, 2012; Oukarroum et al., 2012). To test such a hypothesis experimentally is very difficult, and the specific site of action of a variety of metals which target photosynthesis (e.g. copper, zinc, cadmium, and lead) is still highly contested (Ralph et al., 2007). Despite using different algae, nanomaterials and exposure times, a number of studies have also demonstrated the photoinhibitory effect of silver nanoparticles, and have suggested that blocking the electron transport at the primary electron donor of plastoquinone (Qₐ) is a potential mechanism of toxicity in algae (Dewez and Oukarroum, 2012; Oukarroum et al., 2012, Matorin et al., 2013), and it has been suggested that free radical induced damage to photosystem II and disruption of the oxygen evolving complex drives toxicity (Oukarroum et al., 2012). ΦPSII was chosen in this study for its ease of measurement (Ralph et al., 2007; Navarro et al., 2008), for potential incorporation into standard toxicity tests (see next section); additional parameters require
dark adaptation/alternative methodology (see Dewez and Oukarroum, 2012; Oukarroum et al., 2012 for examples) and may not reflect the true activity of PSII in normal, light adapted conditions. Despite this, it is clear that a range of photosynthetic parameters have great value in determining potential toxic pathways of both silver (Navarro et al., 2008; Miao et al., 2010; Dewez and Oukarroum, 2012; Oukarroum et al., 2012; Matorin et al., 2013) and other metals/metal nanoparticles (Miles et al., 1972; Prasad and Stzralka, 1999; Faller et al., 2005; Ait Ali et al., 2006; Perrault et al., 2011; Behra et al., 2015). Future mechanistic nanomaterial toxicity studies involving algae should not only consider the range of available photosynthetic parameters, but also include both ionic controls and substances of known photoinhibitory toxicity (e.g. DCMU).

5.6.2. Concentration response data: 4 and 24 hour photosynthetic efficiency inhibition

In addition to the 30 minute continuous exposure toxicity studies, ΦPSII inhibition was measured within the context of the standard OECD 201 algal growth inhibition test (OECD, 2011; Chapter 3). This was chosen for two reasons; continuous 30 minutes exposures lacked the sensitivity to generate EC_{50} data (even at extremely high silver concentrations), and short term (< 24 hour) photosynthetic efficiency inhibition has previously been demonstrated to be in good agreement with 72 hour growth inhibition in a range of chemical toxicants (Masojidek et al., 2011). EC_{50} data have also been successfully generated from other photosynthetic parameters/test systems at time points < 24 hours, but not compared to growth inhibition (Snel et al., 1998; Petersen and Kusk, 2000; Juneau et al., 2002; 2003). Furthermore short term testing (< 24 hours) may mitigate potential variability in exposure concentration due to test system-toxicant interactions in longer term (> 24 hours) silver nanoparticle exposures (Sorensen and Baum, 2015).

Assessment of photosynthetic efficiency at 4 and 24 hours proved a suitable endpoint for comparisons with 72 hour growth inhibition tests for silver, although sensitivity was lower in ΦPSII tests. With DCMU however, ΦPSII tests were of equal or greater sensitivity to 72 hour growth inhibition, depending on cell density measurement method used. These findings were consistent with previous work investigating photosynthetic efficiency inhibition in relation to growth inhibition for herbicides (DCMU, Atrazine, and Isotproturon; Masojidek et al., 2011). Similarly to growth inhibition, ΦPSII inhibition can be measured in both flasks (presented in this chapter) and 24 well plates (Appendix 6). Whilst ΦPSII inhibition by both silver ions and nanoparticles was similar to 72 hour growth inhibition studies in both flasks (Chapter 3) and 24 well plates (Chapter 4), the sensitivity of ΦPSII tests was lower (evidenced by higher EC_{50} values in concentration response studies). A similar reduced ΦPSII test sensitivity was
observed in humic acid and pH studies, with lower ΦPSII inhibition in comparable growth inhibition test conditions containing toxicant and humic acid/pH modification. Despite this, similar patterns in silver toxicity were observed between both endpoints, such as reduction in toxicity over time and with increasing SRHA concentration, and enhanced toxicity at pH 8 compared to unbuffered/pH 6 medium. As such patterns in toxicity has been discussed in detail in Chapters 3 and 4, this discussion will focus instead on the suitability of ΦPSII as a potential alternative endpoint. Short term (< 24 hours) photosynthetic parameters have been demonstrated to predict toxicity of a number of toxicants (such as herbicides, metals, surfactants, and various aromatic compounds), at a similar sensitivity to the standard 72 hour growth inhibition tests (Petersen and Kusk, 2000; Radix et al., 2004; Masojidek et al., 2011). However the lack of standard protocol for photosynthetic 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 ΦPSII is investigated more widely when investigating impacts of substances on algae. Whilst 24 hour exposure time appeared to show slightly better predictive ability than 4 hour (i.e. inhibition results were more similar to growth inhibition data at 72 hours), testing a range of exposure times in the future may reveal a more suitable timepoint. Juneau et al. (2001) found that photosynthetic parameters’ sensitivity varied between toxicants. Therefore further work is needed to determine the suitability of the methods presented here with other nanomaterials and where necessary, their ionic counterparts, as well as a wider range of chemicals.

The suitability of short term photosynthetic efficiency inhibition as an endpoint in more complex media (with humic acids and/or pH modification) has also been demonstrated in this study. Suwannee River humic acid decreased the photosynthetic toxicity of both forms of silver, and pH 8 OECD medium induced a higher photosynthetic toxicity than either unbuffered medium or medium buffered to pH 6. These findings are consistent with results from previous chapters (see Chapters 3 and 4). As ΦPSII was derived fluorimetrically, the same use of appropriate cell free blanks are necessary when testing substances which may interfere with measurements (e.g. nanomaterials, humic substances, etc.), as described in Section 4.6.3. There is potential for humic substances to interfere with the assay used to quantify ΦPSII activity. Other authors have noted the potential for humic acid’s strong fluorescence signal (at ~470nm) to generate false negative inhibition in both Fv/Fm and ΦPSII measurements (Ralph et al., 2007; Schreiber et al., 2007; Schmitt-Jansen and Altenburger, 2008). Although slight humic-enhancement of fluorescence signal at 470 nm was observed, this effect was very small, and not detected at the other wavelengths used in this study (645 and 665 nm). A large increase in ΦPSII was observed in samples containing SRHA at 540 nm, which led to the exclusion of this
wavelength from the data analysis. The fluorescence of humic acids appears to be composition-dependent (Zepp et al., 1981), so future work must carefully consider which wavelengths are selected, and studies must always assess interference from humic acid. The advantage to using multiple wavelength PAM machines is that such interference can be corrected on a post hoc basis. MOPS buffer has a long history of use in algal tests and has no reported interference with photosynthetic endpoints (Ghirardi et al., 2002; Navarro et al., 2008; Choi et al., 2012). As such it had little effect on ΦPSII measurements in this study. Similarly for NM300K, a small increase in fluorescence at the different PAM wavelengths was observed, but such signals were factored out of the results due to the use of cell-free blanks.

The results presented in this chapter highlight both the advantages and disadvantages of ΦPSII. As a toxic endpoint, it is easily measured with very little sampling time required. The 30 minute endpoint presented here demonstrated a toxic effect of both DCMU and silver over a much shorter time period than required for growth inhibition studies. However the sensitivity of the 30 minute endpoint was much lower than the later 4 and 24 hour endpoints (especially in the case of silver). Furthermore, as evidenced by the K2Cr2O7 results, ΦPSII is a more specific endpoint than growth inhibition so may only be of use in studies with toxicants of known photosynthetic effect to avoid the generation of false-negative results. By comparison, algal growth is a much more general endpoint, which may be affected by deleterious toxic effects on a range of biochemical processes. The results of the comparative work presented here indicate that ΦPSII may be more suitable as a battery of specific tests (which could include other photosynthetic endpoints, ROS generation, esterase activity, etc) used to determine the mechanism of toxicity, whereas growth inhibition may be more suitable as it is currently used in regulatory testing.

5.7 Conclusions

The data presented in this chapter show the value of short term photosynthetic assays in algal toxicity tests. Short term (30 minute) continuous assays revealed some similarity between patterns of toxicity of silver (nanoparticles and ionic forms) and DCMU, which requires further investigation. Such tests can also be used to demonstrate a lack of effect for certain toxicants (e.g. K2Cr2O7) and can inform mechanisms of toxicity when compared to growth inhibition studies. By using such reference toxicants and endpoints, stronger conclusions can be made regarding mechanistic explanations of toxicity.

Although results presented here suggest similarities between photosynthetic and growth endpoints for silver (ionic and nanoparticulate) and DCMU, further work is required to assess the suitability of this method to other toxicants. Depending on proposed mechanism of
toxicity, such photosynthesis measurements are easy to obtain and appear to correlate well with longer term growth inhibition. Unlike growth inhibition tests these results cannot be obscured by mortality effects (i.e. >100% inhibition), as only the photosynthesis of viable cells is measured. From a regulatory perspective ΦPSII inhibition may be useful in future tests as its measurement requires less sample preparation and shorter exposures. Short term exposures may be especially useful in nanoparticle research, or with other toxicants suspected to behave dynamically over the standard 72 hour test duration. Other approaches to assess various aspects of photosynthetic activity are available and although they were not tested in this study, they should be compared in future work in an attempt to harmonise test protocols.

Similarly to the work presented in Chapter 4 on well plate derived growth inhibition, photosynthetic inhibition tests can also be carried out in well plates (Appendix 6) which allows this endpoint to be integrated into miniaturised high throughput testing strategies. It is therefore recommended that assessment of photosynthetic activity in toxicity testing is more widely investigated, across a variety of methods, in order to obtain valuable toxicology data for algae/higher plants.
Chapter Six - Final discussion

The work presented in this thesis studied the toxicity of silver nanoparticles (NM300K) to *R. subcapitata* using the OECD 201 Algal Growth Inhibition Test. In addition the toxicity of ionic silver (AgNO$_3$), and two standard reference toxicants (DCMU and K$_2$Cr$_2$O$_7$) were also investigated in the same test system, for comparison. The main aims of this research were to investigate how changes in the test system, such as media, organic matter and pH, affected fate and effects of the different silver chemicals studied. Furthermore, potential modifications to the standard test design were investigated and recommendations made with a view to increasing testing efficiency and speed of testing via the use of smaller test volumes or employment of alternative endpoints.

In Chapter 2, the dissolution, hydrodynamic diameter, zeta potential and polydispersity of NM300K were assessed in different algal growth media. Due to the demonstration of stable NM300K suspensions in a variety of media in other published studies (Klein *et al.*, 2011; Wang *et al.*, 2012; Kermanizadeh *et al.*, 2013; Voelker *et al.*, 2013; Sorensen and Baun, 2015, Donnellan *et al.*, 2016) and questions regarding the toxicological importance of silver nanomaterial solubility (Hwang *et al.*, 2008; Jung *et al.*, 2008; Xiu *et al.*, 2011, 2012; Gunsolus *et al.*, 2015; Navarro *et al.*, 2015), dissolution was chosen as the principal characteristic for detailed investigation. NM300K was found to exhibit low dissolution in all media without Suwannee River humic acid. In these media, dissolution was generally lower at 0 hours than at 72 hours indicating the slow dissolution of silver nanomaterials which has been observed in other studies with different nanomaterials (Metcalf and Harriott, 1972; Moore and Codella, 1998; Liu and Hurt, 2010; He *et al.*, 2012; Xiu *et al.*, 2012). Dissolution was also slightly higher at pH 6 than at pH 8, supporting observations of increased dissolution in more acidic media (Liu and Hurt, 2010; Oukarrum *et al.*, 2014). Investigations into the effect of media on DLS derived NM300K characteristics (hydrodynamic diameter, zeta potential, and PDI) showed little effect, which was attributed to the dominance of steric stabilisation, as NM300K nanoparticle suspensions contain two dispersants (polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan mono-laurat (Tween 20)). Furthermore, the minimum required nanoparticle concentration required for DLS measurements (and indeed, dissolution using AAS) was 50-100 times higher than toxicologically relevant to *R. subcapitata* (growth inhibition EC$_{50}$; established in Chapter 3), so these findings have limited generalisability.

Unexpectedly, Suwannee River humic acid resulted in extremely high NM300K dissolution in both media over 72 hours. These findings are far in excess than those found in published literature on silver nanomaterial dissolution in media containing humic substances.
(Liu and Hurt, 2010; Gao et al., 2012; Zhang et al., 2012; Pokhrel et al., 2014; 2013; Gunsolus et al., 2015), which calls into question the suitability of the ultracentrifugal nanoparticle separation method used in this work (Section 2.4.2.1.) with regards to its ability to fully separate out NM300K in samples containing humic acid. Further work is needed to verify these dissolution studies. If dissolution rates in media containing SRHA are truly representative, one may expect either a considerable reduction in number or size of silver nanoparticles in a given sample. Such a reduction could be detected using TEM with EDX, or dissolved silver could be measured in situ using ion selective electrodes in order to cross validate ICP-MS work.

Finally, the limited study on dissolved silver in freshly opened and one year old vials supports the representative test material concept for NM300K; silver dissolution between the two vials were remarkably similar despite the loss of the inert argon packaging atmosphere in the year old vial, provided to reduce oxidative dissolution (Liu and Hurt, 2010; Klein et al., 2011). Limited investigation into ICP-MS silver recovery (i.e. total particulate and dissolved fractions) tentatively suggested a reasonable recovery in 0 hour samples, but a reduction in 72 hour samples (mainly from a loss of particulate fraction). Given the sample processing time required, it was not possible to determine recovery in all samples, so the recovery data presented here should be interpreted with caution. In future work it may be valuable to quantify recovery in all samples, especially as loss of silver to the test system has been demonstrated in both glass (Sekine et al., 2015) and plastic (Lee et al., 2004; Malysheva et al., 2015). Such losses may be the cause of time dependent reduction in silver toxicity observed (Chapters 3, 4 and 5). In addition, ICP-MS data presented here were obtained from algal cell free media, and so may not be truly representative of the dynamic chemical processes exhibited in actively growing algal cultures. As algae grow they modify the growth medium so that factors such changes in pH, nutrient depletion and EPS production over the duration of testing remain largely unquantified with respect to their effect on silver (both ionic and nanoparticulate). As mentioned previously, ion selective electrodes may help to quantify silver speciation over the duration of algal growth inhibition tests and help to account for potential losses of silver to the test system.

The aim of Chapter 3 was to assess the toxicity of NM300K and AgNO₃ to R. subcapitata under standard OECD 201 test design (OECD, 2011). In addition, the effect of deviations from the standard protocol (using non-standard test medium) on silver toxicity were considered, and the impact of pH and presence of humic acid assessed evaluate environmental relevancy. The OECD 201 test appears to be suitable for determining the toxicity of NM300K to R. subcapitata, however it should be considered that NM300K is an example of a well dispersed nanoparticle suspension and other suspensions of nanomaterials
with higher aggregation/agglomeration rates may be less well suited to such a test system. Nanomaterials have been documented affecting reproducibility in the OECD 201 test, including both silver (Sorensen and Baun, 2015) and TiO₂ (Hartmann et al., 2010). Despite this, general patterns of silver toxicity (i.e. ionic toxicity > nanoparticulate toxicity, reduction in toxicity over the test duration) in the work presented in Chapter 3 were consistent with published studies.

Due to the relative flexibility of the OECD 201 test requirements with respect to media choice, two test media were chosen in which to study comparative silver toxicity; OECD 201 medium and JM. Dissolution studies in Chapter 2 revealed that mean NM300K dissolution was slightly greater in JM than in OECD medium. This difference in NM300K dissolution may partially explain the increased toxicity in JM relative to OECD medium. However in ionic studies this pattern of toxicity was reversed, with a greater toxicity of AgNO₃ in OECD medium compared to JM. In an attempt to account for this difference, and informed by studies of the effect of various silver species on toxicity (Lee et al., 2004; Roemer et al., 2011; Tejamaya et al., 2012; Zhang et al., 2012; Seo et al., 2014; Mousavi et al., 2015), speciation was modelled using Visual Minteq and dissolved silver data from Chapter 2. Although silver toxicity was higher in JM for NM300K both in terms of total mass and ICP-MS derived dissolved silver, it was lower when expressed as modelled free Ag⁺. This was attributed to a combination of higher dissolved silver and less chloride in JM compared to OECD. Chloride reduces silver toxicity (Lee et al., 2004) and is in much higher concentration in OECD medium, which may explain reduced toxicity in this medium. However for AgNO₃ toxicity was higher in OECD medium in terms of both total mass/dissolved, and when expressed as modelled free Ag⁺. This suggests that whilst Ag⁺ may be a determining factor in silver toxicity, other factors (speciation, differences in ionic and nanoparticulate silver, etc.) may be responsible as observed by the differences in media once Ag⁺ was accounted for. Given that speciation modelling was carried out on cell free dissolved silver measurements from Chapter 2, further work is needed to establish and quantify speciation over the duration of a standard algal toxicity test. Regardless of further study, this work reveals the importance of using the standard medium when following the OECD 201 test protocol to ensure valid comparisons between laboratories. In addition, and especially in the case of nanomaterials where the relative importance of ionic release is contested, the use of ionic controls and assessment of dissolution should be considered standard practice in order to address their contribution to observed toxicity in parallel to studies with nanomaterials.

The addition of humic acid reduced silver toxicity in both media in a concentration dependent manner, a phenomena which has been observed in a range of test systems/organisms (Fabrega et al., 2009; Gao et al., 2012; McLaughlin and Bonzongo, 2012;
Zhang et al., 2012; Kim et al., 2013; Gunsolus et al., 2015; Mousavi et al., 2015). When Suwannee River humic acid was accounted for in modelled silver speciation calculations, it became evident silver-humic complexation increased with increasing concentrations of humic acid, primarily through the relative depletion of free Ag⁺. However, and somewhat unexpectedly, the addition of higher concentrations of Suwannee River humic acid lowered toxicity, but prevented the time dependent reduction observed in other test conditions (i.e. no increase in EC₅₀ was observed over the 72 hour test duration). Such an observation may have important consequences for humic acid’s contribution to the persistence of sublethal concentrations of silver in natural environments, and warrants further investigation. Suwannee River humic acid represents only a small part of the highly heterogeneous family of biogenic compounds classified as natural organic matter. It has been demonstrated that other types of natural organic matter differ in their chemical composition, and so exert differing effects on the characteristics and resultant toxicity of both silver nanomaterial suspensions (Gunsolus et al., 2015) and ionic solutions (Mousavi et al., 2015). Contextualising the effect of silver nanomaterials and their ionic counterparts within more environmentally relevant test systems is an important and promising field of research, from which a number of future projects could be generated. Such projects may serve to inform future regulatory framework and help identify vulnerable ecosystems.

Despite a higher NM300K dissolution in both media buffered to pH 6 (relative to both unbuffered media and media buffered to pH 8), toxicity tended to be higher for both forms of silver in media buffered to pH 8. When silver speciation was calculated in both OECD medium and JM buffered to pH 6 and 8, a similar pattern was observed in both media. Media buffered to pH 6 caused a relative increase in the less toxic AgCl(aq) species and a reduction in Ag⁺, whereas in pH 8 media the opposite effect was observed. If Ag⁺ is the dominant source of toxicity to R. subcapitata (Lee et al., 2004; 2005), the results presented here suggest that reduction in the toxicity of silver may be primarily driven by changes in speciation which deplete Ag⁺ in favour of less toxic species (AgCl(aq) in the case of differences between media and pH, and Ag-humic complexes in the case of Suwannee River humic acid). As with the ICP-MS dissolution studies presented in Chapter 2, modelled silver speciation is subject to the same limitations regarding algal chemical modification to the test medium during active growth. Future studies, where possible, should address speciation experimentally through the use of ion selective Ag⁺ electrodes to quantify silver partitioning at multiple timepoints during toxicity testing (see Gunsolus et al., 2015 and Mousavi et al., 2015 for examples of studies with silver nanoparticles and ions respectively). In addition, such work would be of great value in determining the degree to which silver nanoparticle toxicity can be explained on the basis of
dissolved ions (which to date still remains an actively debated research question). Furthermore more strict control of pH, and the understanding of chemical speciation within the test system, have been identified as two areas of improvement in the current OECD 201 test guidelines.

In both humic acid and pH studies with NM300K, difficulties were experienced in obtaining stable toxicity measurements. In this work, a NM300K concentration was chosen based on probit modelled results from Section 3.5.1 and 3.5.2., in order to induce a 72 hour growth inhibition effect of ca. 80-100% in humic acid free, unbuffered test medium. This concentration was calculated to be 190 µg/l NM300K in OECD medium and 80 µg/l NM300K in JM. These test conditions were used as controls (i.e. humic acid free and unbuffered), meaning that growth inhibition could be directly compared between studies in both humic acid and pH studies. For the same NM300K concentration in OECD medium (190 µg/l), the control 72 hour growth inhibition was higher in humic acid studies than in pH studies. The same difference in relative control toxicity was observed in JM, containing 80 µg/l NM300K. The reason for the variability in NM300K toxicity between different studies can be explained in a number of ways. Firstly, the JRC specifically recommend not re-using vials after opening, and that silver nanoparticles may agglomerate and settle out of suspension over time (Klein et al., 2011). Secondly, different vials of nanoparticles were used for different experiments as only 2 ml of nanoparticle suspension is provided in each vial, which resulted in vials being quickly emptied, making the requirement for additional vials an inevitability. The different vials used could vary in their toxicity due to inter-batch variability, although the JRC nanomaterial repository specifically attempts to reduce such variation. A similar issue was not observed for AgNO₃, as there was good reproducibility between humic and pH studies in both media which suggests that the algae did not vary in their sensitivity over time. Attempts were made to pinpoint the source of variation observed, for example by testing dissolution between new and old vials (Section 2.5.1.2). To address the variability in NM300K toxicity some authors have recommended ageing nanoparticle suspensions for 24 hours before adding algae, to allow suspensions to reach equilibrium (Sorensen and Baum, 2015). Using such a strategy may be useful to reduce variability, but would make comparisons with published studies more difficult as such a protocol is rarely used at present.

Estimations of growth inhibition based on probit modelling overestimated toxicity (i.e. predicted growth inhibition > observed growth inhibition) for AgNO₃ in OECD medium, and underestimated toxicity in JM. Most concentration response models are designed to give EC₅₀ data with the highest degree of precision, with uncertainty increasing as effective concentration estimates move away from this value (i.e. towards EC₀ or EC₁₀₀). To address this uncertainty in future work, a full concentration response curve could be generated for all
levels of humic acid/pH. In this work such an approach was impractical due to the space and time constraints required for fully replicated studies in flasks. However with the use of miniaturised toxicity testing presented in Chapter 4, such an approach may be more practical in future studies. In addition, such an experimental design has the value of assessing the effect of a fixed pH or humic acid level over the whole concentration response curve, which would determine whether any changes in toxicity are constant or dependent on toxicant concentration.

In Chapter 4, the importance of test vessel and algal enumeration method was considered in silver studies, under the same experimental conditions as presented for OECD medium in Chapter 3. Namely, the effect of assessing toxicity in a miniaturised, high-throughput, 24 well plate test design was considered for its suitability for increasing the speed with which nanomaterial toxicity can be screened. For use with this test design, a simple in vivo Chl\(_a\) fluorescence cell density measurement method was presented which minimises sample processing time and correlates well with more time consuming and variable cell counting. Whilst R. subcapitata 72 hour growth rate was slightly lower in 24 well plates compared to flasks, they satisfied the OECD 201 test validity requirement for a growth rate of 0.92 d\(^{-1}\) (equivalent to a 16 fold increase in cell density).

In addition to the two forms of silver (NM300K, AgNO\(_3\)), concentration response data was also generated for the photosynthetically active herbicide DCMU. Across these three toxicants the miniaturised test design was at least as sensitive as the algal toxicity data (72 hour growth inhibition EC\(_{50}\)) generated in 250 ml Erlenmeyer flasks (NM300K), or in some cases more sensitive (i.e. lower EC\(_{50}\); AgNO\(_3\) and DCMU). However test sensitivity was considered to be primarily driven by R. subcapitata growth measurement method, as toxicity data generated in both well plates and flasks were more similar when the same measurement was used (in vivo Chl\(_a\)) compared to when different measurement methods were used in well plates (in vivo Chl\(_a\)) compared to flasks (in vitro Chl\(_a\)). Due to the destructive nature of the in vitro Chl\(_a\) method, and the limited volume in 24 well plates, this method was deemed unsuitable for miniaturised test systems. Despite this, robust toxicity data was generated in flasks in Chapter 3 using in vitro Chl\(_a\). In addition, humic and pH studies were carried out in 24 well plates for comparison with data collected in Chapter 3. Despite some differences in absolute toxicity (percentage growth inhibition), patterns of toxicity were broadly similar to those described in Chapter 3; namely, higher toxicity of AgNO\(_3\) compared to NM300K, time dependent reduction in silver toxicity, a reduction in toxicity with increasing concentration of Suwannee River humic acid, and increased toxicity in OECD medium buffered to pH 8 compared to unbuffered medium or medium buffered to pH 6.
Whilst flasks are considered the standard test design in most algal toxicity studies, the OECD 201 test does not explicitly forbid the use of smaller test volumes provided validation criteria are met. One criteria which was difficult to verify in 24 well plate toxicity tests was pH change over the test duration. This was due to the fact that such small volumes (2 ml per test chamber) made pH measurements difficult. OECD test medium has good intrinsic buffering capacity due to the presence of NH\textsubscript{4}\textsuperscript{+} which offsets algal alkalisation of the test medium through the liberation of protons (H\textsuperscript{+}) during nitrogen assimilation (See Section 3.5.1.2. for evidence of tight pH control in flask based studies); it therefore could be argued that the buffering capacity of OECD medium could be assumed in the 24 well plate design. Despite this future studies should attempt to verify this hypothesis through the use of micro pH meters designed specifically for measuring pH in small volumes (e.g. Yamada et al., 2010).

Finally, the results presented in Chapter 4 should not be generalised beyond the toxicants used. Whilst similar fluorometric methods have been used to determine the toxicity of silver (Sekine et al., 2015), titanium dioxide (Hunde-Rinke and Simon, 2006; Lee and An, 2013) and zinc oxide (Lee and An, 2013) nanoparticles to algae, such methods require further validation to ensure they are widely applicable to a range of nanomaterials, and that nanomaterials do not interfere with fluorometric methods of cell density estimation. In flask based studies using acetone extracted \textit{in vitro} Chl\textsubscript{a} methods (Chapter 3), locust gum was added to aid the precipitation and sedimentation of nanoparticles and avoid potential interfering fluorescence. The results presented in this thesis offer a method that allows both miniaturised and flask based toxicity tests to be conducted in parallel, allowing comparisons between methods used with other toxicants (including, but not limited to nanomaterials) relatively straightforward. If such miniaturised methods are found to be suitable across a range of toxicants, they would be valuable tool in regulatory ecotoxicology, making considerable savings in sample processing time, increasing statistical replication, and allow multiple toxicants to be tested in parallel. Such benefits have the potential to begin the automisation of algal growth inhibition tests, with a view towards high-throughput screening of a variety of toxicants.

The aim of Chapter 5 was to investigate and compare the sublethal effects of silver (NM300K and AgNO\textsubscript{3}), DCMU and K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} on \textit{R subcapitata} short term (< 30 minutes) photosynthetic yield (\textgreek{\Phi}_{PSII}). Using this endpoint, 30 minute EC\textsubscript{50} data could only be generated for DCMU which is likely due to the fact that it was specifically synthesised to be a fast-acting photosynthetically active herbicide (Miles, 1990). By contrast, after 30 minutes of exposure, concentrations of both NM300K (0-1000 µg/l Ag) and AgNO\textsubscript{3} (0-500 µg/l Ag) could not be used to generate \textgreek{\Phi}_{PSII} inhibition EC\textsubscript{50} data. Interestingly for silver, both NM300K and AgNO\textsubscript{3}
reached an inhibitory plateaux where higher concentrations did not result in a further increase in ΦPSII inhibition; in NM300K this was observed at 500 µg/l Ag and for AgNO₃ it was 100 µg/l Ag. Comparatively, K₂Cr₂O₇ (0-100 mg/l) did not result in any ΦPSII inhibition over the 30 minute exposure time, despite the highest concentration tested being around 100 times greater than the EC₅₀ in equivalent growth inhibition studies (Appendix 3). Whilst such a test may not be able to generate concentration response data for all toxicants, by using comparative toxicants of known photosynthetic mechanisms of action (e.g. DCMU), more information can be gained on the potential mechanism of action in the toxicant of interest (in this thesis, silver). Once introduced to the test system, silver appears to act quickly on ΦPSII in a similar manner to DCMU, indicating that it is to some extent a photosynthetically active toxicant. However from the inhibitory plateaux effect on ΦPSII observed in silver, there may be rate limiting effects on the uptake of silver which limit its short term toxic effect. This could be verified through the addition of salts to the test medium, known to modify silver uptake rate in algae, such as chloride or thiosulphate (Fortin and Campbell, 2001; Kramer et al., 2002; Lee et al., 2004; 2005; Hiriart-Baer et al., 2006). Establishing the precise site of silver’s toxic impact on photosynthesis is experimentally more difficult however. Furthermore, the evidence provided here does not address the possibility of additional sites of silver’s toxic impact that are not related to photosynthesis (Oukarroum et al., 2012).

Once the photoinhibitory effect of silver was established, the same endpoint was investigated at later timepoints (4 and 24 hours) for NM300K, AgNO₃ (Chapter 3) and DCMU (Chapter 4). Correlations were made between 4 and 24 hour ΦPSII inhibition and 72 hour growth inhibition to identify the relationship between photosynthetic efficiency and growth rate. One advantage of these experiments was that photosynthetic inhibition data could be easily generated, simply by taking small-sample (1.5-2 ml) measurements from ongoing toxicity tests (i.e. suitable for use with both flasks and 24 well plate systems), without the need for a separate experimental design. As a result, direct comparisons could be made between growth- and photo-inhibition without the errors associated with separating the test systems. It was determined that both 4 and 24 hour ΦPSII was a good indicator of 72 hour growth rate, and so may potentially be used as a shorter term endpoint for determining silver toxicity. When this endpoint was deployed in humic acid and pH studies, similar results were found.

Despite the utility of this endpoint for silver, future studies should first establish if the toxicant of interest is photosynthetically active (K₂Cr₂O₇ is an example of a toxicant for which such an endpoint is not suitable), before attempting to establish correlations with later growth inhibition endpoints. Other measures of photosynthetic activity could also be assessed, in order to determine the most valuable photosynthetic parameter to study across a range of
toxicants, or to establish which (if any) part/s of the photosynthetic process an individual toxicant may act upon.

In summary, the work presented in this thesis has provided information on the toxicity of silver nanomaterials to *R. subcapitata* and the suitability of applying the OECD 201 test to accomplish this. In addition, it has revealed the importance of performing such tests in well-defined media and attempted to add some environmental relevancy to these results through the addition of humic acid and modification of test medium pH. Media choice was found to play an important role in modifying toxicity, and as such, the choice of test medium in algal toxicity tests must not be considered trivial. Where possible, the effect of various test medium compounds on the toxicant of interest must be well understood/described in future studies. In addition, humic acid and pH modification has been demonstrated to modify silver (ionic and nanomaterial) toxicity which has important relevance to both the hazard of silver in natural systems (humic acid, pH), and to potential changes in silver toxicity over the duration of algal growth inhibition tests (through changes in pH).

A miniaturised alternative test system has been proposed which may improve ecotoxicological output for laboratories with limited resources, and shown how a photosynthetic endpoint can be used to gain some insight into silver’s mechanism of toxicity. Both miniaturised test systems and the alternative photosynthetic endpoint showed comparable patterns in silver and DCMU toxicity, but differed in their sensitivity. These findings have drawn attention to important areas of further study. These include how to generate reliable characterisation data at concentrations that are toxicologically relevant, and finding better ways to quantify silver ions in ongoing concentration response tests in order to establish their relative contribution to algal toxicity. Furthermore, there is a need for greater environmental relevancy in toxicology data, to establish the true threat of predicted increases in products containing silver nanomaterials in the natural environment, and which ecosystems may be at particular risk. Finally silver’s toxic mechanism of action in algae is still poorly understood, so future studies may be useful to identify the specific sites of photosynthesis which silver act upon, and establish any addition sites of toxic impact on algal physiology.

The conclusions and recommendations made in this thesis are made upon data generated from limited set of toxicants. As such, future studies should test the extent to which these findings may be applied to other toxicants (including nanomaterials), species and test conditions.
Appendices

Appendix 1 - Media recipes

Stock solutions were prepared in 250 ml glass bottles, by adding the appropriate mass of compound to 18 mΩ water. Stock solutions were sterilised by autoclaving for 15 minutes at 121°C, except for those containing heat sensitive compounds (NaHCO$_3$, EDTA salts and vitamins). For such stock solutions, filter sterilisation was used (0.2 µm pore diameter). Stocks were stored in the dark at room temperature until ready to use.

Table A1.1: OECD 201 medium – pH ~7.8

<table>
<thead>
<tr>
<th>Stock</th>
<th>Component</th>
<th>Stock Concentration</th>
<th>Stock Concentration</th>
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</tr>
<tr>
<td>3A</td>
<td>H$_3$BO$_3$</td>
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<td></td>
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</tr>
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<td>CoCl$_2$.6H$_2$O</td>
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<tr>
<td>4</td>
<td>NaHCO$_3$</td>
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</tr>
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</table>
### Table A1.2: Jaworsky’s medium (JM) – pH ~7.0

<table>
<thead>
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<th>Stock</th>
<th>Component</th>
<th>Stock Concentration</th>
<th>mg/l (Stock)</th>
<th>mg/l (Final)</th>
<th>ml/l</th>
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<td>Ca(NO$_3$)$_2$.4H$_2$O</td>
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<tr>
<td>2</td>
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<td>Biotin</td>
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<tr>
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</table>
Appendix 2 - SOP for humic acid and pH modification to standard OECD 201 test

Proposed: 1 June 2017

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Addition to OECD 201 Freshwater Alga and Cyanobacteria, Growth Inhibition Test

Standard Operating Procedure for testing the effect of humic acid and pH on the toxicity of substances to algal growth inhibition

INTRODUCTION

1. The purpose of the OECD 201 test is to determine the effect of a substance on the growth of freshwater microalgae and/or cyanobacteria, over a test period of usually 72 hours (1). As part of the OECD Guidelines for the Testing of Chemicals, it serves as part of a battery of tests designed to assess the potential effect of chemicals on human health and the environment, and is a tool used for industrial, academic and governmental decision making.

2. This additional SOP aims to contextualise the toxicity data generated by the OECD 201 test within a broader environmental perspective, by investigating two dynamic environmental factors - humic acid and pH. Understanding how toxicity of a substance is modified by these factors may aid in the decision making process, by helping to identify ecosystems which may be vulnerable to a given substance, and planning risk mitigation strategies as appropriate.

3. As a supplemental test guideline, this SOP is informed directly by, and uses terminology defined in, the OECD 201 test (1). Modifications to the original test guidelines are stated herein under the same headings; otherwise refer to referenced paragraphs (in italics) in the original OECD 201 test guideline (1).

PRINCIPLE OF THE TEST

4. In order to deploy this SOP correctly, robust concentration response data must first be generated under the requirements of the OECD 201 test guidelines (1). As an additional test, this SOP can be deployed using the same apparatus, test organisms, and growth medium as used in the OECD 201 test. Modifications to the humic acid content and pH presented in this SOP, should be constrained within those found in typical natural systems (0-50 mg/l humic acid; pH 6 - pH 8) and also by the tolerances of the chosen test species (usually Raphidocelis subcapitata).

5. This test is designed to investigate the effect of two levels of humic acid and two pH levels on the toxic effect of a single concentration of substance (predicted to induce a 72 hour growth inhibition of 80%). It situations where full concentration response curves are required for humic acid and pH, it is advised that the OECD 201 test guidelines be followed, for each level of the medium modification of interest.

INFORMATION ON THE TEST SUBSTANCE

6. Refer to OECD 201 test guidelines (1) (Paragraphs 9-10).

VALIDITY OF THE TEST

7. Refer to OECD 201 test guidelines (1) (Paragraph 11).

8. In addition, the testing of humic acid and pH must be conducted in the same medium as used to determine concentration response data for the results to be valid.

REFERENCE SUBSTANCE

9. 3,5-dichlorophenol and potassium dichromate, as recommended in OECD 201 test guidelines (1) (Paragraph 12).

10. Concentrations predicted to inhibit 72 hour algal growth by 80% should be determined for previous reference substance testing, and used in this SOP.

APPLICABILITY OF THE TEST

11. Refer to OECD 201 test guidelines (1) (Paragraph 13).
DESCRIPTION OF THE METHOD

12. Refer to OECD 201 test guidelines (1) for apparatus (Paragraphs 14-16) and test organisms (Paragraphs 17-18).

Growth medium

13. The recommended growth medium (OECD and AAP (1), Paragraph 19). Note that due to the differences in media composition, the two should not be used interchangeably (See “Validity of the test”). Three separate growth media are necessary - one without modification, and one for each level of humic acid/pH. It is important (especially in the case of pH studies) to allow growth media to equilibrate with atmospheric conditions to ensure stable pH. This can be achieved by bubbling with filtered air for 30 minutes.

14. Modification of humic acid can be achieved by adding humic acid to the required concentration of interest, directly into prepared growth medium.

15. Modification of pH requires the use of buffers. OECD 201 guidelines allow a pH drift of < 1.5 units (1), but in tests where pH is investigated a more conservative drift (< 0.5 units) should be prioritised. The choice of buffer is ultimately the result of balancing a range of competing practical considerations such as: toxicity to test species, target pH, potential complexation with test substance/medium components, and environmental relevancy (a selection of suitable buffers can be found in (2)(3)). Buffers should be added to growth media and adjusted to target pH using hydrochloric acid and/or sodium hydroxide. Once algae and/or test substance have been added to pH adjusted media, further pH adjustment may be necessary before taking measurements.

Initial biomass concentration

16. Refer to OECD 201 test guidelines (1) (Paragraph 21).

Concentration of test substance

17. A single test substance concentration which induces 80 % growth inhibition over 72 hours should be determined from previous OECD 201 test results. Note that estimating 80 % inhibitory concentration may require extrapolation from original concentration response results, so further concentration response data may be required to reliably estimate 80 % inhibitory concentration.

Replicates and controls

18. The test design should include at least three replicates at each test condition. These are:

   Master controls (no test substance or medium modification)
   Humic/pH control one (no test substance)
   Humic/pH control one (no test substance)
   Unmodified test substance (80 % inhibitory concentration, no medium modification)
   Humic/pH modification + test substance one (80 % inhibitory concentration)
   Humic/pH modification + test substance one (80 % inhibitory concentration)

19. At least two levels of humic acid/pH modification should be selected. If a greater number of humic acid/pH levels are desired, each must have both control and test substance treatments. The number of control replicates should be at least three (preferably six for master controls).

20. Separate test solutions for analytical determination of test substance concentrations and solvent controls should be deployed as necessary, according to OECD 201 protocol (1) (Paragraphs 24-25).

Preparation of inoculum culture

21. Refer to OECD 201 test guidelines (1) (Paragraph 26).
Preparation of test solutions

22. Refer to OECD 201 test guidelines (1) (Paragraphs 27-28).

Incubation

23. Refer to OECD 201 test guidelines (1) (Paragraphs 29-31).

24. With respect to Paragraph 30 of the OECD 201 test guideline (1), the more conservative pH drift of < 0.5 units should be prioritised in tests where pH is investigated.

Test duration

25. Refer to OECD 201 test guidelines (1) (Paragraph 32).

Measurements and analytical determinations

26. Refer to OECD 201 test guidelines (1) (Paragraphs 33-40).

27. In pH tests, daily measurement of pH is preferred. If a suitable buffer is chosen, daily pH adjustment should not be necessary.

Other observations

28. Refer to OECD 201 test guidelines (1) (Paragraph 41).

DATA AND REPORTING

Plotting growth curves

29. Refer to OECD 201 test guidelines (1) (Paragraphs 43-45).

30. Compare growth curves for both master controls and controls with humic acid/pH modification. Clearly identify any differences in growth between master and modified controls.

Response variables

31. As in OECD 201 test guidelines (1) (Paragraph 46), both average specific growth rate and yield can be used to compare humic/pH effect on toxicity. Response variables chosen must be the same as used to previously determine concentration response relationships.

Average growth rate

32. Refer to OECD 201 test guidelines (1) (Paragraphs 48-49).

33. When calculating growth inhibition from average growth rate (1) (Paragraphs 50-51), calculate inhibition of both humic acid/pH controls relative to master controls in order to determine any inhibitory effect of humic acid/pH alone. Calculate test substance treatment inhibition to their corresponding controls (i.e. with or without humic acid/pH modification).

Yield

34. When calculating growth inhibition from yield (1) (Paragraph 52), follow the same procedure of comparison between treatments as described in “Average growth rate”, but substituting yield.

Plotting differences in growth inhibition as a result of humic acid/pH

35. Plot percentage inhibition for each humic acid/pH treatment. Examine percentage inhibition in unmodified test medium and ensure approximately 80 ± 10 % inhibition is achieved. Failure to achieve target inhibition may indicate poor concentration response curve fit around the EC_{80} value (i.e. high uncertainty), or poor concentration range selection in original OECD 201 test.

Statistical procedures

36. Comparison of test substance effect between levels of humic acid/pH treatment can be compared using analysis of variance techniques (ANOVA), as for determining LOEC/NOEC in the OECD 201 test guidelines (1) (Paragraph 57). ANOVA assumptions of normal distribution and homogeneity of variance can be tested with Levene’s or Bartlett’s tests. Transformation (e.g. logarithmic) can be used in
circumstances where ANOVA assumptions are invalidated. If transformation results in data that continue to invalidate ANOVA assumptions, non-parametric statistics such as Kruskal-Wallis and Mann Whitney U tests can be used. Growth inhibition in control treatments without test substance should also be compared in this manner, to investigate the effect of humic acid/pH treatment alone.

**Test report**

37. In addition to the reporting requirements of the OECD 201 test guideline (1) (*Paragraph 61*):

**Test substance:**

- As described in OECD 201 test guidelines (1) (*Paragraph 61*).
- Predicted 80% inhibition concentration.
- Observed experimental inhibition.

**Humic acid/pH modifications:**

- Selected humic acid concentrations.
- Type and source of humic acid (including IHSS catalogue number if used).

*AND/OR*

**pH modification:**

- Selected pH range.
- Type and source of buffer (including rationale for choice).
- Quantities and concentrations of HCl and/or NaOH added to buffer stocks.
- Quantities and concentrations of buffer stocks added to treatments.
- Quantities and concentrations of HCl and/or NaOH added to treatments.

**Test conditions:**

- As described in OECD 201 test guidelines (1) (*Paragraph 61*).

**Results:**

- As described in OECD 201 test guidelines (1) (*Paragraph 61*), without concentration/effect relationship and response variables.
- pH values at the beginning and at the end (or every day for pH tests) of the test at all treatments.
- Graphical presentation of changes in growth inhibition across humic acid/pH treatments.
- ANOVA results indicating significant changes in test substance induced growth inhibition as a result of humic acid/pH treatment.
- ANOVA results indicating significant changes in humic acid/pH control growth inhibition relative to master controls.

**Literature**


Appendix 3 - $K_2Cr_2O_7$ toxicity in OECD test medium (OECD 201 test)

**Figure A3.1**: Impact of potassium dichromate ($K_2Cr_2O_7$) [0.03-10 mg/l] on *R. subcapitata* growth rate inhibition in OECD medium, at 24 hours (black circles) 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean ($n = 1$, one experiment with three replicates).

**Table A3.1**: Probit modelled effective concentrations of $K_2Cr_2O_7$ [0.03-10 mg/l] on *R. subcapitata* growth rate (24, 48, and 72 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model of $K_2Cr_2O_7$ induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses ($n = 1$, one experiment with three replicates). A One way ANOVA between each timepoint found no significant differences ($p = 0.618$) in EC$_{50}$ value.

<table>
<thead>
<tr>
<th>$K_2Cr_2O_7$ (mg/l)</th>
<th>24 hour</th>
<th>48 hour</th>
<th>72 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.77 (±0.04)</td>
<td>0.80 (±0.05)</td>
<td>0.83 (±0.05)</td>
</tr>
<tr>
<td>20</td>
<td>1.07 (±0.04)</td>
<td>1.13 (±0.04)</td>
<td>1.14 (±0.04)</td>
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<tr>
<td>50</td>
<td>1.64 (±0.05)</td>
<td>1.77 (±0.05)</td>
<td>1.74 (±0.05)</td>
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<tr>
<td>80</td>
<td>2.21 (±0.07)</td>
<td>2.40 (±0.07)</td>
<td>2.34 (±0.07)</td>
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Appendix 4 - DCMU toxicity in OECD test medium (OECD 201 test)

Figure A4.1: Impact of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [0.2-100 µg/l] on *R. subcapitata* growth rate inhibition in OECD medium, at 24 hours (black circles) 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Table A4.1: Probit modelled effective concentrations of DCMU [0.2-100 µg/l]) on *R. subcapitata* growth rate (24, 48, and 72 hour) in OECD medium. Data expressed as mean effective concentration derived from a probit model of DCMU induced growth inhibition (compared to toxicant free control). Standard error of the mean is shown in parentheses (n = 1, one experiment with three replicates Superscript letters denote significant differences in EC$_{50}$ value between each timepoint (One way ANOVA; p < 0.05); EC$_{50}$ values not sharing the same superscript letter were significantly different.

<table>
<thead>
<tr>
<th>DCMU (µg/l)</th>
<th>24 hour</th>
<th>48 hour</th>
<th>72 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5.53 (±2.54)</td>
<td>2.01 (±2.04)</td>
<td>2.89 (±1.82)</td>
</tr>
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<td>20</td>
<td>25.52 (±1.97)</td>
<td>17.59 (±1.63)</td>
<td>16.73 (±1.47)</td>
</tr>
<tr>
<td>50</td>
<td>63.76 (±2.01)$^a$</td>
<td>47.42 (±1.49)$^a$</td>
<td>43.20 (±1.36)$^c$</td>
</tr>
<tr>
<td>80</td>
<td>102.01 (±3.32)</td>
<td>77.24 (±2.23)</td>
<td>69.67 (±1.99)</td>
</tr>
</tbody>
</table>
INTRODUCTION

38. The purpose of the OECD 201 test is to determine the effect of a substance on the growth of freshwater microalgae and/or cyanobacteria, over a test period of usually 72 hours (1). As part of the OECD Guidelines for the Testing of Chemicals, it serves as part of a battery of tests designed to assess the potential effect of chemicals on human health and the environment, and is a tool used for industrial, academic and governmental decision making.

39. This additional SOP aims to increase the efficiency of the OECD 201 test, by describing a method designed for use with smaller volumes (24 well plates) than the recommended flask based approach. No change in test chamber is necessary, and the reduced volume allows a far greater number of substances/test concentrations/replicates to be investigated in parallel.

40. As a modified test guideline, this SOP is informed directly by, and uses terminology defined in, the OECD 201 test (1). Modifications to the original test guidelines are stated herein under the same headings; otherwise refer to referenced paragraphs (in italics) in the original OECD 201 test guideline (1).

PRINCIPLE OF THE TEST

41. As a modified test protocol, this SOP can be deployed using the same apparatus, test organisms, and growth medium as used in the OECD 201 test.

42. This test is designed to improve the efficiency of the OECD 201 test protocol through a combination of smaller test vessels (24 well plates) and non-destructive sampling (chlorophyll a fluorescence).

INFORMATION ON THE TEST SUBSTANCE

43. Refer to OECD 201 test guidelines (1) (Paragraphs 9-10).

VALIDITY OF THE TEST

44. Refer to OECD 201 test guidelines (1) (Paragraph 11).

REFERENCE SUBSTANCE

45. 3,5-dichlorophenol and potassium dichromate, as recommended in OECD 201 test guidelines (1) (Paragraph 12).

APPLICABILITY OF THE TEST

46. Refer to OECD 201 test guidelines (1) (Paragraph 13).

DESCRIPTION OF THE METHOD

47. With modification to OECD 201 test guideline (1) (Paragraph 14), tests should be conducted in disposable sterile 24 well plates, so prewashing should not be necessary. Well plates which have been treated for tissue culture are unsuitable for use in this protocol due to demonstrated inhibition of algal growth (2). Recommended test vessels should be untreated, transparent, flat-bottomed polypropylene.

48. With modification to OECD 201 test guideline (1) (Paragraph 15), the needs for sufficient gas exchange should be balanced against the need to avoid evaporation of small volumes in wells. This can be achieved through sealing of well plates with moisture-impermeable/gas-permeable film (e.g. Parafilm M®), and incubating well plates on a rotary shaker. Liquid volumes should be 1.5 ml per well.
49. With modification to OECD 201 test guideline (1) (Paragraph 16), non-destructive sampling techniques should be used. Growth in well plates should be measured with a fluorometric plate reader (see Paragraph 24 of this protocol).

50. Refer to OECD 201 test guidelines (1) for test organisms (Paragraphs 17-18).

Growth medium

51. Refer to OECD 201 test guidelines (1) (Paragraphs 19-20).

Initial biomass concentration

52. Refer to OECD 201 test guidelines (1) (Paragraph 21).

Concentration of test substance

53. Refer to OECD 201 test guidelines (1) (Paragraph 22).

Replicates and controls

54. Refer to OECD 201 test guidelines (1) (Paragraph 23-25). Whilst a standard test with replication can be conducted in 24 well plates, multiple well plates can be deployed when increased replication/additional concentrations are desired.

Preparation of inoculum culture

55. Refer to OECD 201 test guidelines (1) (Paragraph 26).

Preparation of test solutions

56. Refer to OECD 201 test guidelines (1) (Paragraphs 27-28).

Incubation

57. Refer to OECD 201 test guidelines (1) (Paragraphs 29-31).

58. With modification to OECD 201 test guideline (1), plates should be sealed with moisture-impermeable/gas-permeable film (e.g. Parafilm M®) to prevent evaporation.

Test duration

59. Refer to OECD 201 test guidelines (1) (Paragraph 32).

Measurements and analytical determinations

60. Refer to OECD 201 test guidelines (1) (Paragraphs 33-40).

61. With modification to OECD 201 test guideline (1) (Paragraph 34). Growth in well plates should be measured with a fluorometric plate reader, set to appropriate excitation emission settings to detect the highest chlorophyll signal. For tests with Raphidocelis (formerly Pseudokirchneriella) subcapitata an excitation and emission of 435 nm and 690 nm has been found to be suitable, but other spectra may be necessary for other test organisms.

62. With modification to OECD 201 test guideline (1) (Paragraph 35). Measurement of pH should be conducted with electrodes specifically designed for use with well plates, as electrodes designed for larger volumes may remove considerable volume from wells during measurement.

Other observations

63. Refer to OECD 201 test guidelines (1) (Paragraph 41).

DATA AND REPORTING

Plotting growth curves

64. Refer to OECD 201 test guidelines (1) (Paragraphs 43-45).
Response variables
65. Refer to OECD 201 test guidelines (1) (Paragraphs 46-47).

Average growth rate
66. Refer to OECD 201 test guidelines (1) (Paragraphs 48-51).

Yield
67. Refer to OECD 201 test guidelines (1) (Paragraph 52).

Plotting concentration response curve
68. Refer to OECD 201 test guidelines (1) (Paragraph 53).

Statistical procedures
69. Refer to OECD 201 test guidelines (1) (Paragraphs 54-58).

Test report
70. Refer to OECD 201 test guideline (1) (Paragraph 61):

Literature

Appendix 6 - Photosynthetic inhibition of silver in 24 well plates

Figure A6.1: Impact of AgNO$_3$ [0.2-20 µg/l Ag] on R. subcapitata photosynthetic efficiency ($\Phi$PSII) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean ($n = 1$, one experiment with three replicates).
Figure A6.2: Impact of NM300K [30-150 µg/l Ag] on R. subcapitata photosynthetic efficiency (ΦPSII) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean (n = 3).
Figure A6.3: Impact of NM300K [190 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 3).
Figure A6.4: Impact of AgNO$_3$ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on R. subcapitata photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. AgNO$_3$ photosynthetic inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates).
**Figure A6.5**: Impact of NM300K [190 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 3).
Figure A6.6: Impact of AgNO₃ [6 µg/l Ag] and pH (6 and 8; buffered using 3.5mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates).
Table A6.1: The impact of AgNO₃ [6 µg/l] and NM300K [190 µg/l] on short term (4 and 24 hours) photosynthetic inhibition (ΦPSII), compared to longer term (24, 48, and 72 hours) growth inhibition (measured using both in vivo and in vitro Chlₐ methods; see chapters 3 and 4) in *R. subcapitata* in OECD medium with different levels of Suwannee River humic acid (0, 5 and 50 mg/l). Standard error of the mean in parentheses.

<table>
<thead>
<tr>
<th>Endpoint</th>
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<th>NM300K + 5 mg/l SRHA</th>
<th>NM300K + 50 mg/l SRHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosynthetic inhibition</td>
<td>190 µg/l</td>
<td>190 µg/l + 5 mg/l SRHA</td>
<td>190 µg/l + 50 mg/l SRHA</td>
</tr>
<tr>
<td>4 h</td>
<td>88.70 (±1.06)</td>
<td>84.17 (±1.07)</td>
<td>-0.12 (±3.61)</td>
</tr>
<tr>
<td>24 h</td>
<td>54.61 (±2.46)</td>
<td>22.57 (±2.50)</td>
<td>2.22 (±1.03)</td>
</tr>
<tr>
<td>Growth inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vivo</em> Chlₐ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>661.05 (±96.95)</td>
<td>513.91 (±82.94)</td>
<td>-6.27 (±13.03)</td>
</tr>
<tr>
<td>48 h</td>
<td>171.93 (±4.26)</td>
<td>147.75 (±6.15)</td>
<td>47.86 (±4.26)</td>
</tr>
<tr>
<td>72 h</td>
<td>96.82 (±4.34)</td>
<td>84.08 (±2.57)</td>
<td>53.70 (±4.05)</td>
</tr>
<tr>
<td><em>AgNO₃</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photosynthetic inhibition</td>
<td>6 µg/l</td>
<td>6 µg/l + 5 mg/l SRHA</td>
<td>6 µg/l + 50 mg/l SRHA</td>
</tr>
<tr>
<td>4 h</td>
<td>80.00 (±0.11)</td>
<td>75.58 (±0.39)</td>
<td>5.44 (±1.31)</td>
</tr>
<tr>
<td>24 h</td>
<td>38.60 (±1.40)</td>
<td>21.81 (±0.73)</td>
<td>-0.70 (±0.22)</td>
</tr>
<tr>
<td>Growth inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vivo</em> Chlₐ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>180.09 (±3.28)</td>
<td>127.95 (±5.04)</td>
<td>54.08 (±1.61)</td>
</tr>
<tr>
<td>48 h</td>
<td>128.26 (±5.35)</td>
<td>104.24 (±7.90)</td>
<td>62.40 (±4.15)</td>
</tr>
<tr>
<td>72 h</td>
<td>79.51 (±4.80)</td>
<td>59.40 (±6.17)</td>
<td>37.02 (±4.53)</td>
</tr>
</tbody>
</table>
Table A6.2: The impact of AgNO$_3$ [6 µg/l] and NM300K [190 µg/l] on short term (4 and 24 hours) photosynthetic inhibition (ΦPSII), compared to longer term (24, 48, and 72 hours) growth inhibition (measured using both in vivo and in vitro Chl$_a$ methods; see chapters 3 and 4) in *R. subcapitata* in OECD medium with different levels of pH modification (unbuffered, pH 6 and pH 8). Standard error of the mean in parentheses.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>NM300K</th>
<th>NM300K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>190 µg/l unbuffered</td>
<td>190 µg/l pH 6</td>
</tr>
<tr>
<td>Photosynthetic efficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>71.00 (±7.95)</td>
<td>64.92 (±11.26)</td>
</tr>
<tr>
<td>24 h</td>
<td>51.06 (±8.62)</td>
<td>56.21 (±15.47)</td>
</tr>
</tbody>
</table>

Growth inhibition

**In vivo Chl$_a$**

<table>
<thead>
<tr>
<th></th>
<th>190 µg/l unbuffered</th>
<th>190 µg/l pH 6</th>
<th>190 µg/l pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>196.83 (±37.82)</td>
<td>230.44 (±47.49)</td>
<td>341.96 (±32.82)</td>
</tr>
<tr>
<td>48 h</td>
<td>107.60 (±24.34)</td>
<td>127.41 (±28.36)</td>
<td>182.65 (±15.20)</td>
</tr>
<tr>
<td>72 h</td>
<td>55.89 (±16.61)</td>
<td>91.68 (±25.56)</td>
<td>122.94 (±15.05)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>6 µg/l unbuffered</th>
<th>6 µg/l pH 6</th>
<th>6 µg/l pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>70.62 (±0.41)</td>
<td>66.82 (±1.68)</td>
<td>87.29 (±0.74)</td>
</tr>
<tr>
<td>24 h</td>
<td>26.49 (±0.43)</td>
<td>42.22 (±4.10)</td>
<td>71.50 (±2.89)</td>
</tr>
</tbody>
</table>

Growth inhibition

**In vivo Chl$_a$**

<table>
<thead>
<tr>
<th></th>
<th>6 µg/l unbuffered</th>
<th>6 µg/l pH 6</th>
<th>6 µg/l pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>178.07 (±6.45)</td>
<td>167.23 (±5.05)</td>
<td>197.61 (±6.32)</td>
</tr>
<tr>
<td>48 h</td>
<td>96.63 (±3.47)</td>
<td>100.51 (±3.61)</td>
<td>135.15 (±7.55)</td>
</tr>
<tr>
<td>72 h</td>
<td>40.62 (±3.88)</td>
<td>41.40 (±3.80)</td>
<td>79.87 (±7.57)</td>
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</tbody>
</table>
References


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