Ecotoxicity of nanomaterials in relation to the freshwater microalga *Raphidocelis subcapitata.*

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Abstract

Silver is the most frequently represented nanomaterial in available products, ranging across a variety of commercial and medical goods. Their main justification for inclusion in these products relates to their unique properties compared to both their bulk (larger particulate) and ionic equivalents. It is established that silver is highly toxic to a range of aquatic organisms, and despite its existence in low concentrations in the environment, concerns have been raised over products containing silver nanomaterials as a potential emerging pollutant. Within the EU the environmental risk assessment of chemicals is regulated by the Registration, Evaluation and Authorization of Chemicals (REACH) regulation, and toxicity testing deployed according to a series of tiered production/import thresholds. At the lowest regulatory production/import threshold of one tonne, the Organisation for Economic Co-operation and Development (OECD) 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test can be deployed to evaluate the toxicity to the aquatic environment via assessment of the impact of chemicals on algal species, over a 72 hour test duration. Due to the unique physico-chemico properties of nanomaterials, there is considerable uncertainty over the suitability of such test protocols for these materials. Thus, there may be a requirement for alternative test systems. In addition, the proliferation of the nanotechnology industry has created a diverse range of materials whose safety needs to be assessed. There is therefore an immediate need for rapid environmental risk assessment in order to keep pace with commercial activities. This thesis addresses such considerations in four stages. First, the OECD 201 test was applied to evaluate the toxicity of a representative silver nanoparticle (20-150 μg/l Ag as NM300K, at 24-72 hours) and $AgNO_3$ (0.2-30 μg/l Ag, at 24-72 hours), following dispersion in two different growth media, and the influence of different medium conditions such as humic acid (through the use of environmentally relevant concentrations of Suwannee River humic acid, [5 and 50 mg/l] and pH (6 and 8) on silver nanoparticle toxicity was investigated. In addition, a miniaturised version of the OECD 201 test was developed and compared to the standard test method, as a means to increase the rate and efficiency at which laboratories can generate toxicity data. Finally an alternative, short term sublethal photosynthetic endpoint was investigated in order to identify an alternative measure of toxicity to algae and inform the toxic mechanism of action of silver to algae, and correlations with the standard growth inhibition endpoint presented. It was found that the OECD 201 test was suitable to evaluate toxicity in NM300K and enabled identification of a 72 hour growth inhibition effective concentration (EC_{50}) of 54-130 μg/l Ag. Of interest was that silver nanoparticle toxicity decreased with exposure duration and were affected by test medium composition. $AgNO_3$ toxicity was higher than NM300K (72 hour EC_{50} = 5-7 μg/l Ag)., but subject to the same time and media effects Humic acid decreased the toxicity of both forms of silver in a

concentration dependent manner, and pH 8 test medium increased toxicity relative to pH 6. The proposed miniaturised test was found to be as sensitive as the standard method (for NM300K) across a similar range of test conditions. Finally, the short-term photosynthetic sublethal endpoint indicated that silver may act in a similar way to known phototoxic substances, and by measuring this endpoint a high correlation was found with later 72 hour growth inhibition. Obtained data therefore demonstrate that the OECD 201 test can be used with *R. subcapitata* as a tool to assess nanoparticle toxicity, and that there are opportunities to increase the efficiency of testing via miniaturisation of the test system and the use of additional toxicity endpoints.

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I would like to thank my two supervisors, Professor Teresa Fernandes and Doctor Helinor Johnston, for giving me the opportunity to perform this research and for their support and understanding during my PhD studies.

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"We take a handful of sand from the endless landscape of awareness around us and call that handful of sand the world."

— Robert M. Pirsig (1974), Zen and the Art of Motorcycle Maintenance: An Inquiry into Values

DECLARATION STATEMENT

(Research Thesis Submission Form should be placed here)

List of Tables and Figures

List of tables

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

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 t_0 dissolution variation was identified as an artefact of sample preparation (due to batch preparation of samples); therefore such treatments may not represent true t_0 values. This may also explain the apparent relative reduction in time dependent dissolution in JM pH 8, as an artefact.

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 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 EC_{50} between each timepoint (Kruskal-Wallis; $p < 0.05$); EC_{50} values not sharing the same superscript letter were significantly different.

Table 3.2: Probit modelled effective concentrations of $AgNO_3$ [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 EC_{50}, but a Mann-Whitney U test was unable to identify specific differences.

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.

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.

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_{50} between media, at each timepoint (One way ANOVA; $p < 0.05$); EC_{50} not sharing the same superscript letter were significantly different.

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).

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).

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 EC_{50} between media, at each timepoint (24 hours: Mann-Whitney U; 72 hours: One way ANOVA; $p < 0.05$); EC_{50} not sharing the same superscript letter were significantly different.

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 $AgNO_3$ (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 EC_{50} between media, at each timepoint (One way ANOVA; $p < 0.05$); EC_{50} not sharing the same superscript letter were significantly different.

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. $HAAg_{(aq)}$ denotes a soluble monodentate complex of humic acid and silver, whereas $HA-Ag^+G_{(aq)}$ 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 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. $HAAg_{(aq)}$ denotes a soluble monodentate complex of humic acid and silver, whereas $HA-Ag^+G_{(aq)}$ 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 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 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 3.14: 72 hour growth inhibition of NM300K and AgNO$_3$ 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 AgNO$_3$ 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 3.15: 72 hour growth inhibition of NM300K and AgNO$_3$ 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 (n_{NM300K} = 3; n_{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 3.16: Silver (ionic and nanoparticles) EC$_{50}$ values to a variety of algal species. EC$_{50}$ 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 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 AgNO$_3$ [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.

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 AgNO$_3$ [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.

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 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 flasks *in vivo*.

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 4.5: Probit modelled effective concentrations of $AgNO_3$ [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 $AgNO_3$ 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 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.

Table 5.1: Common fluorimetrically-derived photosynthetic parameters (adapted from Consalvey *et al.*, 2005 and Ralph *et al.*, 2007)

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 5.3: Probit modelled effective concentrations of $AgNO_3$ [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 $AgNO_3$ 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 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 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 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 5.7: 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 A1.1: OECD 201 medium – pH ~7.8

Table A1.2: Jaworsky's medium (JM) – pH ~7.0

Table A3.1: Probit modelled effective concentrations of K$_2$Cr$_2$O$_7$ [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$_2$Cr$_2$O$_7$ 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 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 A6.1: 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 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.

List of figures

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 nanowhisker 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).

Figure 1.2: Light microscope image of *R. subcapitata* in OECD medium. Bar = 20 μm

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.

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.

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.

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.

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).

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.

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.

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.

Figure 2.11: TEM image of NM300 sample directly from vial (concentration - 100 g/l). Taken from Klein *et al.* (2011). Timepoint is not specified.

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 ($n_{OECD\ medium}$ = 92; n_{JM} = 63). Letters denote significant differences within each timepoint (Mann-Whitney U; $p < 0.05$); bars not sharing the same letter were significantly different.

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).

Figure 3.3: Impact of AgNO$_3$ [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).

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.

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.

Figure 3.6: Impact of AgNO$_3$ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* growth inhibition, in OECD medium. AgNO$_3$ 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.

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).

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).

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 ($n_{control}$ = 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.

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.

Figure 3.11: Impact of pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on $AgNO_3$ [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.

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).

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).

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 ($n_{control}$ = 22; 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.

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.

Figure 3.16: Impact of $AgNO_3$ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* growth inhibition, in OECD medium. $AgNO_3$ 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.

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).

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.

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.

Figure 3.21: Impact of $AgNO_3$ [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.

Figure 4.1: Change *R. subcapitata* cell density (starting density 5×10^4 cells/ml) in cultures grown in OECD medium, as determined by a) manual cell counts, b) optical density at 685 nm, c) *in vitro* Chl_a, d) *in vivo* Chl_a (Ex/Em: 435/685 nm). Data expressed as mean, and standard error of the mean (n = 3)

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* Chl$_a$ (µg/l) and *in vivo* Chl$_a$ (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.

Figure 4.3: Relationships between a series of cell density surrogate measures and cell counts: a) optical density vs cell counts; b) *in vitro* Chl$_a$ vs cell counts; c) *in vivo* Chl$_a$ vs cell counts (cells/ml); for *R. subcapitata* cultures grown in OECD medium. (n = 30)

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.

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$_a$ 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 and standard error of the mean (six replicates); 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 (µ) in OECD medium between the 24 well plate test and the OECD standard method (conducted in flasks, *in vitro* Chl$_a$ 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 and standard error of the mean (six replicates); 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 4.7: 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* Chl$_a$) 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 and standard error of the mean (six replicates); 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}).

Figure 4.8: Impact of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [0.2-100 µg/l] on *R. subcapitata* growth rate inhibition at 72 hours in OECD medium, in flasks/*in vivo* Chl$_a$ (black circles), flasks/*in vitro* Chl$_a$ (grey squares) and 24 well plates/*in vivo* Chl$_a$ (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).

Figure 4.9: Impact of NM300K [30-150 µg/l] on *R. subcapitata* growth rate inhibition at 72 hours in OECD medium, in flasks/*in vivo* Chl$_a$ (black circles), flasks/*in vitro* Chl$_a$ (grey squares) and 24 well plates/*in vivo* Chl$_a$ fluorescence (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 2).

Figure 4.10: Impact of AgNO$_3$ [0.2-20 µg/l] on *R. subcapitata* growth rate inhibition at 72 hours in OECD medium, in flasks/*in vivo* Chl$_a$ (black circles), flasks/*in vitro* Chl$_a$ (grey squares) and 24 well plates/*in vivo* Chl$_a$ 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).

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).

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.

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.

Figure 4.14: Impact of AgNO₃ [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.

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* 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 pH condition (One way ANOVA/Kruskal-Wallis; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 4.16: 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.

Figure 4.17: Impact of AgNO₃ [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.

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 AgNO₃ [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 $AgNO_3$ [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_a extraction) for $AgNO_3$ [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_a and 24 well plates using *in vivo* Chl_a (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$_a$) for AgNO$_3$ [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 flasks using *in vivo* Chl$_a$ (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.

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 (Q_a), and then to the secondary plastoquinone acceptor (Q_b), where it remains. Simultaneously, the oxidised P680 is reduced by the enzymatic photolysis of water (H_2O) into oxygen ($1/2O_2$), protons ($2H^+$) and electrons (e^-). An additional photon excites another single electron, which proceeds along the same pathway as the first to Q_b. The now fully reduced Q_bH_2 transfers the electron pair through cytochrome b$_6$f 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).

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).

Figure 5.3: Impact of $K_2Cr_2O_7$ [0-100 mg/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. Data expressed as mean ΦPSII and standard error of the mean (n = 1, one experiment with three replicates).

Figure 5.4: Impact of $AgNO_3$ [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 = 3).

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).

Figure 5.7: Impact of $AgNO_3$ [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).

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).

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 photosynthetic inhibition (compared to toxicant free controls) 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.

Figure 5.10: 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). Letters denote significant differences within each timepoint (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

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

Figure 5.12: Impact of $AgNO_3$ [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.

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).

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).

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).

Figure A6.1: Impact of $AgNO_3$ [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).

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 AgNO$_3$ [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).