Freshwater dispersion stability of PAA-stabilised cerium oxide nanoparticles and toxicity towards *Pseudokirchneriella subcapitata*

Andy Booth*[1], Trond Størseth[1], Dag Altin[2], Andrea Fornara[3], Anwar Ahniyaz[3], Harald Jungnickel[4], Peter Laux[4], Andreas Luch[4], Lisbet Sørensen[1]

[1]SINTEF Materials and Chemistry, Trondheim N-7465, Norway

[2]BioTrix, Trondheim N-7022, Norway

[3]German Federal Institute for Risk Assessment (BfR), Department of Product Safety, Berlin, Germany

[4]SP Chemistry, Materials and Surfaces, Drottning Kristinas vag 45, SE-11686, Stockholm, Sweden

*Corresponding author: andy.booth@sintef.no, +47 93089510

Abstract

An aqueous dispersion of poly (acrylic acid)-stabilised cerium oxide (CeO_2) nanoparticles (PAA-CeO_2) was evaluated for its stability in a range of freshwater ecotoxicity media (MHRW, TG 201 and M7), with and without natural organic matter (NOM). In a 15 day dispersion stability study, PAA-CeO_2 did not undergo significant aggregation in any media type. Zeta potential varied between media types and was influenced by PAA-CeO_2 concentration, but remained constant over 15 days. NOM had no influence on PAA-CeO_2 aggregation or zeta potential. The ecotoxicity of the PAA-CeO_2 dispersion was investigated in 72 h algal growth inhibition tests using the freshwater microalgae *Pseudokirchneriella subcapitata*. PAA-CeO_2 EC_{50} values for growth inhibition (GI; 0.024 mg/L) were 2-3 orders of magnitude lower than pristine CeO_2 EC_{50} values reported in the literature. The concentration of dissolved cerium (Ce^{3+}/Ce^{4+}) in PAA-CeO_2 exposure suspensions was very low, ranging between 0.5-5.6 µg/L. Free PAA concentration in the exposure solutions (0.0096-0.0384 mg/L) was significantly lower than the EC_{10} growth inhibition (47.7 mg/L) value of pure PAA, indicating free PAA did not contribute to the observed toxicity. Elemental analysis indicated up to 38% of the total Cerium becomes directly associated with the algal cells during the 72 h exposure. TOF-SIMS analysis of algal cell wall compounds indicated three different modes of action, including a significant oxidative stress response to PAA-CeO_2 exposure. In contrast to pristine CeO_2 nanoparticles, which rapidly aggregate in standard ecotoxicity media, PAA-stabilised CeO_2 nanoparticles remain dispersed and available to water column species. Interaction of PAA with cell wall components, which could be responsible for the observed biomarker alterations, could not be excluded. This study indicates that the increased dispersion stability of PAA-CeO_2 leads to an increase in toxicity compared to pristine non-stabilised forms.

Keywords – nanoparticles; CeO_2; dispersion stability; ecotoxicity; freshwater algae

1. Introduction

Owing to their radical scavenging and UV-filtering properties, cerium oxide (CeO_2) engineered nanoparticles (ENPs) offer a solution to several technological challenges. Currently, major uses include CeO_2 ENP-based catalytic filters to reduce exhaust particle emissions from diesel combustion (Park et al., 2007) and as an antioxidant, protecting biological tissue from oxidative stress induced by reactive oxygen species (ROS) (Karakoti et al., 2008). Other engineering and biological applications of CeO_2 ENPs include solid-oxide fuel cells, high-temperature oxidation protection materials, catalytic materials, solar cells and potential pharmacological agents in bioanalysis, biomedicine, drug delivery, and bioscaffolding (Xu et al., 2014) (and references therein).

Inevitably, CeO_2 ENPs will be released to the aquatic environment, where their fate and potential impacts will depend on their physicochemical properties (size, shape, surface chemistry) and environmental conditions (pH, ionic strength, colloids and natural organic matter (NOM) content) (van Hoecke et al., 2011; Booth et al., 2013). In aqueous environments, CeO_2 ENPs may undergo a variety of transformation processes, including homo-aggregation, settling and dissolution. Interaction with other particulates (hetero-aggregation) or compounds present in the water column may drive the aggregation process or help stabilise dispersed ENPs. Aggregation and sorption behaviour can have a significant effect on ENP toxicity (Adegboyega et al., 2012; Baalousha et al., 2013; Louie et al., 2013).

A study investigating the behaviour of CeO_2 ENPs in different natural waters showed that sedimentation and hetero-aggregation with natural colloids were the main removal mechanisms (Quik et al., 2012). The concentration and composition of NOM in natural waters varies significantly (Wang et al., 2011) and influences ENP behaviour (Keller et al., 2010;

Quik et al., 2010; Quik et al., 2012; Loosli et al., 2013; Gallego-Urrea et al., 2014). Fulvic and humic acids present in NOM can stabilise CeO_2 ENPs in natural waters and in algae growth media, either by electrostatic or steric repulsion (Quik et al., 2010). Furthermore, pH significantly affects NOM adsorption to CeO_2 ENPs, thus influencing CeO_2 ENP aggregate size (van Hoecke et al., 2011). In freshwater and under conditions relevant for ecotoxicological tests CeO_2 ENPs tend to agglomerate, which can have effects on bioavailability and toxicity (Rodea-Palomares et al., 2011; Röhder et al., 2014).

The ecotoxicity of a wide range of unmodified ('pristine') CeO_2 ENPs to aquatic species such as bacteria, algae, zooplankton and fish, has been the subject of many studies (van Hoecke et al., 2009; Johnston et al., 2010; García et al., 2011; Sánchez et al., 2011; Manier et al., 2013; Röhder et al., 2014). However, there still remains limited information on the effects of CeO_2 ENPs to algae. Growth inhibition of the freshwater microalga *Pseudokirchneriella subcapitata* has been reported in different studies over a concentration range of 4.4–29.6 mg L^{-1} CeO_2 ENPs (van Hoecke et al., 2009; Rogers et al., 2010; Manier et al., 2011; Rodea-Palomares et al., 2011; Manier et al., 2013). The measured dissolved cerium(III) concentration in CeO_2 ENPs suspensions was low and therefore not considered to be relevant for toxicity of CeO_2 ENPs (van Hoecke et al., 2009; Rogers et al., 2010; Rodea-Palomares et al., 2011). In most studies CeO_2 ENPs did not form stable dispersions in algal exposure media, undergoing different degrees of agglomeration (Rodea-Palomares et al., 2011). However, primary particle size was found to influence toxicity irrespective of agglomeration, with smaller nominal diameters increasing growth inhibition (van Hoecke et al., 2009).

Importantly, CeO_2 ENP growth inhibition in algae appears to be significantly influenced by the dispersion method and age of the suspension (Manier et al., 2011; Manier et al., 2013). In

all studies, flocculation of algae cells or clustering of CeO_2 ENPs around the cell surface was observed. Direct contact of CeO_2 ENPs with algae can cause membrane damage in *P. subcapitata* and may be responsible for the observed toxicity (Rogers et al., 2010; Rodea-Palomares et al., 2011). Under experimental light conditions, CeO_2 ENPs can generate hydroxyl radicals causing lipid peroxidation (Rogers et al., 2010), whilst an increase in intracellular reactive oxygen species (ROS) in algae has been observed (Rodea-Palomares et al., 2012).

Increasingly, ENP physicochemical properties are being modified in order to improve their performance in different technologies and applications. Stabilising agents to maintain ENPs in aqueous dispersion are becoming common, resulting in the surface of the ENPs being coated by organic compounds (Sehgal et al., 2005; Salazar-Sandoval et al., 2014). There is a need to understand how these modified ENPs behave in the environment and what impacts such modifications have on their toxicity, especially compared to the large body of data available for pristine ENPs. Garcia et al. (García et al., 2011) performed a range of standardised aquatic ecotoxicity tests on CeO_2 ENP dispersions stabilised with hexamethylenetetramine (HMT). The CeO_2 ENPs exhibited high toxicity to *D. magna* (48 h acute LC_{50} was 0.012 mg/mL) and *V. fischeri* (Microtox® bioluminescence inhibition was >80 % at 0.064 mg/mL). The HMT stabiliser was demonstrated not to be toxic in this study, but may play a role in the observed toxicity of the CeO_2 ENPs.

In the current study, the dispersion stability of poly (acrylic acid)-stabilised CeO_2 ENPs (PAA-CeO_2) in a range of common ecotoxicity media and their subsequent ecotoxicity to *P. subcapitata* was assessed. The stability and aggregation of PAA-CeO_2 was studied over time and the influence of Suwannee River NOM (SR-NOM) on dispersion stability was also

investigated. Ecotoxicity of the PAA-CeO_2 and pure PAA to *P. subcapitata* was assessed using a modified version of the algal growth inhibition method (OECD 201) to overcome the problem of algal cell 'shading' by ENPs when measuring algal growth by fluorescence. Changes in the levels of algal cell wall compounds were monitored using TOF-SIMS. Particulate CeO_2 and dissolved Ce^{3+}/Ce^{4+} concentration was determined by ultracentrifugation and HR-ICP-MS analysis. The total Ce (dissolved plus particulate) concentration in selected filtered (no algae present) and non-filtered (algae present) exposure samples was determined using HR-ICP-MS to investigate PAA-CeO_2 uptake/adsorption by the algae.

2. Experimental

2.1. Chemicals and materials

All chemicals were of analytical grade, and deionised water was from a Miele Aqua Purificator C7749 system. Poly acrylic acid (PAA, average MW<1800) was purchased from Sigma Aldrich. Suwannee River NOM (SR-NOM) was purchased from International Humic Substances Society (St. Paul, USA). Medium hard synthetic water (MHRW) was made as according to US EPA 821-R-02-12 (US EPA, 2002), media for freshwater algae (TG 201) was made according to OECD Guideline 201, media for *Daphnia magna* (M7) was made according to OECD Guideline 202. All salts and compounds used in the preparation of media water were of analytical grade and supplied by acknowledged international manufacturers. Finally, the pH of the solutions was adjusted as according to the guidelines.

2.2. CeO_2 nanoparticle synthesis and characterisation

CeO_2 nanoparticles were synthesised by thermolysis of $Ce(NO_3)_4$ at high temperature, resulting in homogenous precipitation of a cerium oxide nanoparticle pulp (Chane-Ching, 1994). To stabilise the CeO_2 nanoparticles in water, PAA was employed as an anionic

146 stabiliser and added in excess (Sehgal et al., 2005). A final PAA-stabilised colloidal
147 dispersion of CeO_2 particles (PAA-CeO_2) in MilliQ water (10% wt., 100 mg/L), with a pH of
148 8.5, was prepared for further study. Relevant physical and chemical characterisation
149 techniques were employed to study the PAA-CeO_2. The zeta potential and average
150 hydrodynamic radius (by volume; dynamic light scattering, DLS) of the stock solution was
151 determined using a Malvern Zetasizer. A Phillips CM30 and a Jeol 2100 Transmission
152 electron microscopes (TEM) operated at 150kV and 120kV respectively, both equipped with a
153 LaB6 electron filament were used to investigate individual PAA-CeO_2 crystallite size and
154 shape. Samples were prepared by adding a droplet of the PAA-CeO_2 stock solution to a holey
155 copper grid and allowing the water to evaporate. The Jeol 2100 was equipped with an INCA
156 (from Oxford Instruments) Energy-dispersive X-ray spectroscope (EDX) which was used to
157 study the elemental composition of the PAA-CeO_2 stock material and identify any significant
158 impurities. For the same purpose, Selected Area Electron Diffraction (SAED) pattern analysis
159 was performed with the CM30.
160
161 *2.3. PAA analysis*
162 The concentration of free/excess PAA in the PAA-CeO_2 stock solution, and any subsequent
163 dilutions, required determination in order to account for any ecotoxicological effect. As the
164 PAA was a complex mixture of poly acrylic acid molecules with an average molecular weight
165 of 1800, an NMR-based analysis and quantification method was used. A pure PAA standard
166 in deionised water was prepared and serially diluted to create a calibration curve (0.01 - 10
167 mg/L). To determine the free PAA concentration in the PAA-CeO_2 stock solution, a sample
168 was diluted in deionised water to a PAA-CeO_2 concentration of 10 mg/L. A 5 mL aliquot was
169 then subjected to ultracentrifugation at 65,000 rpm for 60 min (Sorvall WX Ultra, rotor T-
170 865). The supernatant was collected and analysed to quantify the amount of dissolved PAA

remaining. NMR sample preparation was done by adding 20 µl of D_2O containing 1mM 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) to 180 µl of standard solutions and supernatant from sample centrifugation. ^{1}H-NMR spectra were recorded using a Bruker DRU 600 spectrometer (Bruker BioSpin GMBH, Rheinstetten, Germany) operating at 600.13 MHz for ^{1}H using a 1D NOESY (noesygppr1d) pulse sequence from the Bruker pulse sequence library for suppression of residual water. The region from 3.2-0.5ppm was used for the PAA and this was calibrated against the TSP peak. The PAA concentration of the centrifuged sample was determined by the linear regression equation of the standard curve.

2.4. Particulate CeO_2 and dissolved Ce^{3+}/Ce^{4+} analysis

In order to determine the dissolved Ce^{3+}/Ce^{4+} concentration present in the stock solution, a 10 mg/L dilution was prepared and subjected to ultracentrifugation as described above. The supernatant (0.2 mL) was collected and transferred to an ultra-inert sample tube prior to analysis by high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS; Element 2, Thermoelectric) to determine the Ce^{3+}/Ce^{4+} concentration. Samples were analysed without any pre-treatment except dilution in 0.1 M nitric acid.

2.5. Dispersion stability studies

Moderately hard reconstituted water (MHRW) (US EPA, 2002), TG 201 media (freshwater algae, OECD) (OECD, 2011) and M7 (*Daphnia magna*, OECD) were prepared according to the relevant guidelines using reagent grade chemicals and deionised water. For the experiments investigating the influence of natural organic matter (NOM) on dispersion stability, SR-NOM was dissolved in the media at an initial concentration of 20 mg/L and stirred overnight using a magnetic stirrer. After 1 day of stirring, the media-NOM solutions

were filtered using a Nalgene® filtration unit (0.22 µm pore size) to remove any non-dissolved particulate matter. The total organic carbon (TOC) in the resulting solution was determined as 8-9 mg/L (Sievers 900 Portable Turbo instrument). The specific conductivity of each dispersion media was determined, where M7 = 679.7 µS cm^{-1}, M7-NOM = 666.33 µS cm^{-1}, MHRW = 312.7 µS cm^{-1}, MHRW-NOM = 308.2 µS cm^{-1}, TG201 = 163.6 µS cm^{-1}, and TG201-NOM = 163.4 µS cm^{-1}.

The stock dispersion of PAA-CeO_2 was sonicated immediately prior to sub-sampling to ensure homogeneity of the sample prior to dilution in the different media solutions. Two different nominal start concentrations were included in the study; 1 and 0.01 mg/L – three parallels of each concentration in every media. Immediately prior to the first sampling, the samples were homogenised by sonicating for 10 minutes. After this, the samples were left still for the duration of the experiment. Each sample tube was sampled for particle number measurement (dynamic light scattering, DLS) and surface charge measurement (zeta potential) at day 0, 2, 5, 7, 12 and 15.

2.6. Average particle size and zeta potential measurements

The hydrodynamic particle size distribution and zeta potential of the PAA-CeO_2 suspensions was measured using a Zetasizer Nanorange ZS instrument (Malvern, UK). For the size measurements, a small volume of the sample (~0.5 mL) was diluted with the appropriate media solution in a disposable polystyrene cuvette (2.5 mL). The laser source was 632.8 nm with 173 ° backscatter. The zeta potential was measured on the same solution after transfer to a capillary zeta cell. The measurements were performed with automatically optimised number of runs (10-30).

2.7. Algae ecotoxicity studies

The ecotoxicity of PAA-CeO_2, as well as the toxicity of the pure PAA, was investigated using the freshwater algae *Pseudokirchneriella subcapitata* (clone NIVA-CHL1) in a 72 h static growth inhibition test according to OECD 201 (OECD, 2011). For both test materials, a 72 h range-finding pre-test was conducted with sampling at Day 0 and Day 3 and a tentative EC_{50} value determined. Based on these results, 72 h full tests with sampling at Day 0, 1, 2 and 3 was completed (Day 0 samples were collected immediately after preparation to provide baseline values). The pure PAA stabiliser was tested in 12 mL plastic tubes (sample volume 10 mL), at concentrations of 30, 60, 100, 200, 300, 400, 600, 800, 1000 mg/L. As the exposure solutions were free of particulate material, except for the algal cells, *in vivo* fluorescence from chlorophyll was measured directly on the exposure solutions by inserting the tubes in a Spectrophotometer.

Due to the nature of the tested substances, the standard OECD 201 protocol was modified for the PAA-CeO_2. Exposures were completed in 250 ml Erlenmeyer flasks covered with a beaker during incubation at nominal concentrations of 15 (5.5), 25 (12.6), 40 (16.8), 60 (25.4), 100 (32.7) and 200 (67.5) µg/L; actual concentrations determined by HR-ICP-MS given in parentheses (Table 1). In order to overcome potential issues with shading of the algal cells during quantification of the growth, the standard fluorescence method was replaced with a modified version of the ISO method 'Measurement of biochemical Parameters - Spectrometric determination of the chlorophyll-a concentration (ISO 10260: 1992)'. After completion of the exposure period (72 h) the exposure media (10 mL) was filtered using a 0.7 µm glass fibre filter (Whatman GF/F), and the aqueous phase discarded. The filter was then allowed to dry before being added to a vial containing hot ethanol at 75 °C (10 mL) and the chlorophyll pigments extracted by shaking for 5 min. Once cooled to room temperature, the

sample was filtered again to remove any particulate material and the eluent was transferred to a 4.5 mL cuvette and analysed using fluorometer (Turner TD-700, Turner Designs, US). At Day 0 and Day 3, 2 mL aliquots of the exposure solution (before and after the algal filtration step) were collected and subjected to analysis by HR-ICP-MS to quantify the CeO_2 concentration.

2.8. Biokinetics

The metabolic changes of the cell wall after PAA-CeO_2 exposure algal cells were investigated using TOF-SIMS. 10 µL of the algal exposure solution was pipetted onto a gold wafer, fast frozen in liquid nitrogen and stored at -80°C until the TOF-SIMS analysis was performed. Ion spectra measurements were performed using a TOF-SIMS V instrument (IONTOF GmbH, Münster, Germany) with a 30 keV nano-bismuth primary ion beam source. The ion currents were measured to be 0.5 pA at 5 kHz using a Faraday cup. A pulse of 0.7 ns from the bunching system resulted in a mass resolution that usually exceeded 5000 (full width at half-maximum) at m/z <500 in negative mode. The primary ion dose was controlled below 10^{12} ions cm^{-2} to ensure static SIMS conditions (Thompson et al., 2004; Jungnickel et al., 2005; Haase et al., 2011; Tentschert et al., 2013).

2.9. Statistical analyses and calculations

Algal growth rates were calculated by linear regression in Excel v.14.3.9 for Mac OS X (Microsoft Corp., USA) based on the daily increase in biomass measured as fluorescence. Increase in algal biomass during exposure was calculated in Excel as the integral under the growth curve (OECD, 2002). Prior to calculation of effect concentrations the calculated values were normalised to control performance by calculating the percent inhibition. The software GraphPad Prism v5.0b for Mac OS X (GraphPad Software, San Diego, USA) was

used for calculation of effect concentrations (EC_x) and data plotting in the ecotoxicity bioassays. EC_{50}-values were calculated by performing a non-linear regression with a variable slope on the calculated inhibition in growth rates and biomass production at the end of exposure. Constraints were placed at 0 and 100% effect forcing the effect concentrations to be calculated within this span thus eliminating the effect of any stimulation in growth. Values for EC_{10} were calculated in a similar way by using the log(agonist) vs. response (Find ECanything) function in GraphPad Prism on the same data set with the same constrains, and applying least squares fit, as when calculating the EC_{50}-values.

Statistical analysis of the ToF-SIMS data was performed as described in detail elsewhere (Thompson et al., 2004; Jungnickel et al., 2005; Haase et al., 2011; Tentschert et al., 2013). In brief, the acquired data were binned to 1u. Data processing was carried out with the statistical package SPSS+ (version 12.0.2G) using the mass range between 200 and 1700 mass units to detect significant differences between treated cells at time point 0 and treated cells at time point 3 days. Ions lower than mass 200 were excluded from the study to avoid contaminating ions from salts, system contaminants, and other medium components. Each acquired spectrum was then normalised, setting the peak sum to 100%. A Principal Component Analysis (PCA) was performed using all ions. To show that data sets could be separated with a supervised model from each other a Fisher's discriminant analysis was performed. The performance of the discriminant model was verified by applying the cross-validation procedure based on the "leave-one-out" cross-validation formalism.

3. Results and discussion

3.1. CeO_2 nanoparticle synthesis and characterisation

Key physicochemical parameters of the PAA-CeO_2 stock solution are presented in Table S1 in Supplementary Information. The zeta potential of the PAA-CeO_2 stock solution was determined as -25mV, indicating moderate stability of the particles. The average hydrodynamic radius of the PAA-CeO_2 stock solution (determined by volume using DLS) was determined as 84 nm, with a poly dispersity index of 0.234. The crystallite size of the individual CeO_2 particles was determined using TEM as between 4-10 nm, and generally spherical in shape (Figure S1, Supplementary Information). These results indicate that some degree of aggregation or agglomeration of the PAA-CeO_2 particles had occurred leading to the higher hydrodynamic radius determined by DLS. EDX and SAED analysis was used to investigate the purity of the PAA-CeO_2 stock material (Figure S2, Supplementary Information). EDX analysis of the stock PAA-CeO_2 indicated trace amounts of Au, Co, Na and Cl atoms. SAED pattern analysis confirmed that most of the material consists of CeO_2 nanoparticles (CeO_2 has a cubic unit cell, space group Fm-3m (225) and a = 5.412 Å).

3.2. PAA Analysis

The concentration of pure PAA in the 10 mg/L PAA-CeO_2 stock solution was 6.39 mg/L, which represented (63.9% of the CeO_2 concentration). The concentration of pure PAA in the algae exposure solutions ranged from 0.0096-0.128 mg/L (Table 1).

3.3. Dispersion stability studies

The PAA-CeO_2 did not undergo significant aggregation over 15 d in any media type (Figure 1 A-C). After 15 d, the 0.01 mg/L PAA-CeO_2 dispersions do appear to exhibit larger average hydrodynamic sizes than 1.0 mg/L dispersions, however this could be an artefact of the DLS approach which can be sensitive to differences in analyte concentration. It has been suggested that intensity averaged hydrodynamic sizes from DLS analysis, whilst more frequently

321 reported, often are significantly higher than number averaged sizes (Gallego-Urrea et al.,
322 2014). Furthermore, the backscatter angle of 173° also promotes the size distribution towards
323 lower particle sizes through increased elimination of scattering from larger particles. In the
324 current study, TEM imaging of the PAA-CeO_2 stock solution confirmed that it is indeed the
325 number averaged results from DLS that are the most accurate in terms of size (Figure S3,
326 Supplementary Information). Here the average PAA-CeO_2 particles size ranged between 9.1
327 nm and 24.8 nm for both PAA-CeO_2 concentrations in all media types studied, which
328 corresponds more accurately to the crystallite size determined by TEM (4-10 nm).
329
330 The observed dispersion stability is in contrast to the behaviour of 'pristine' CeO_2 ENPs in
331 algae and other ecotoxicity media, where unstable dispersions and agglomeration are typically
332 observed (van Hoecke et al., 2009; Rogers et al., 2010; Manier et al., 2011; Rodea-Palomares
333 et al., 2011; Manier et al., 2013). Furthermore, there was no significant difference in average
334 hydrodynamic diameter (Figure 1 A-C) and zeta potential (Figure 1 D-F) between samples
335 with and without SR-NOM. Previous studies have reported that increasing concentrations of
336 humic acids and alginate (0 – 5 mg/L) give rapidly increased zeta potential and decreased
337 average hydrodynamic size of metal oxide ENPs, showing that NOM stabilises ENPs in water
338 and prevents aggregation (Loosli et al., 2013). The data in the current study indicate that the
339 stabilising effect of the PAA on the CeO_2 ENPs outweighs any additional contribution from
340 the SR-NOM (Figure 1).
341
342 The zeta potential of the PAA-CeO_2 stock solution was -25 mV (stably dispersed), whilst in
343 all media types investigated a significant decrease was immediately observed at 0 d. This
344 indicates a rapid destabilisation of the dispersion driven by the ionic concentration in the
345 different media types (although no significant aggregation was observed over 15 d; Figure 1

D-F). PAA-CeO_2 concentration did not influence zeta potential significantly in MHRW and M7 media. However, a significant difference between PAA-CeO_2 concentrations of 0.01 and 1.0 mg/L was observed for TG201 media, again indicating media type significantly effects dispersion stability. Both theoretical and experimental results have confirmed that zeta potential is affected not only by the suspension conditions such as pH, temperature, ionic strength, and even the types of ions in the suspension, but also by the particle properties such as size and concentration (Tantra et al., 2010; Wang et al., 2013).

A recent study has also highlighted the significant influence that the presence of phosphate can have on increasing zeta potential and stability of CeO_2 ENP dispersions at pH 7.5 (Röhder et al., 2014). Furthermore, Ce^{3+} showed formation of CePO4(s) in the presence of phosphate. In the current study, the zeta potential data are generated from CeO_2 ENPs coated in PAA and dispersion in a complex aquatic system containing dissolved salts (including phosphate) and NOM. It is suggested that the complex interplay of varying ionic strength between the different media types, the presence of phosphate in the media and the interaction of both PAA and NOM at the particle surface, is influencing the stability.

3.4. Algae ecotoxicity studies

PAA-CeO_2 growth inhibition rate EC_{50} values (0.024 mg/L) and biomass production (0.013 mg/L) indicate observed toxicity results from the CeO_2 ENPs with no toxic contribution from the free PAA (Table 2 and Figure 2). The free PAA concentration in the PAA-CeO_2 exposure solutions ranged between 0.0096-0.128 mg/L, which was significantly lower than the EC_{10} and EC_{50} growth inhibition (47.7 and 168.5 mg/L respectively) and biomass production (34.0 and 94.7 mg/L respectively) values of pure PAA. The data indicate that the free PAA present in the PAA-CeO_2 samples did not contribute to the overall toxicity observed, and that toxicity

371 derived directly from the PAA-CeO_2 particles. In the current study, non-stablised CeO_2 ENPs
372 were unavailable for a direct ecotoxicological comparison with the PAA-CeO_2. However,
373 EC_{50} growth inhibition values over a CeO_2 ENP concentration range of 4.4–29.6 mg L^{-1} have
374 previously been reported in a number of studies for the freshwater microalga *P. subcapitata*
375 (van Hoecke et al., 2009; Rogers et al., 2010; Manier et al., 2011; Rodea-Palomares et al.,
376 2011; Manier et al., 2013). These values can be used to assess the influence of PAA
377 stabilisation on the ecotoxicity of CeO_2 ENPs. The EC_{50} growth inhibition value for PAA-
378 CeO_2 determined in the current study (0.024 mg/L) is 2-3 orders of magnitude lower than
379 literature values for pristine CeO_2 ENPs (4.4–29.6 mg L^{-1}). This indicates that the PAA-
380 stabilised CeO_2 ENPs are significantly more toxic than pristine non-stabilised forms. The
381 increased toxicity of the PAA-CeO_2 compared to both the pure PAA and pristine CeO_2 ENPs
382 indicates that there may be a synergistic effect occurring.
383
384 In these previous studies, the measured dissolved cerium (Ce^{3+}/Ce^{4+}) concentration in CeO_2
385 ENPs suspensions was low and therefore not considered to contribute significantly to the
386 observed toxicity of the CeO_2 ENPs (van Hoecke et al., 2009; Rogers et al., 2010; Rodea-
387 Palomares et al., 2011). In the current study, a non-centrifuged sample (nominally 10 mg/L)
388 was determined to have a total Ce (dissolved and particulate) concentration of 4.88 mg/L. The
389 centrifuged sample (only dissolved Ce^{3+}/Ce^{4+}) had a total Ce concentration of 0.329 mg/L,
390 indicating the dissolved Ce^{3+}/Ce^{4+} content was approximately 6.7%. This corresponds to a
391 dissolved Ce^{3+}/Ce^{4+} exposure concentration range of 0.5-5.6 µg/L (Table 1). This is
392 significantly below the EC_{50} value of dissolved Ce^{3+}/Ce^{4+} determined for *P. subcapitata*
393 (Rodea-Palomares et al., 2011), and therefore does not appear to account for the observed
394 toxicity in the PAA-CeO_2 samples. Due to the Kelvin effect, a higher solubility/dissolution
395 kinetics may be expected for CeO2 ENPs used in this study (4-10 nm) compared to those used

396 in other studies (10-60 nm). Furthermore, it is likely that the ultracentrifugation process is not
397 100% efficient at removing CeO2 ENPs from the sample leading to an overestimate of the
398 dissolved Ce concentration. Although the dissolved Ce concentrations are relatively low, they
399 are certainly not negligible. It would therefore be of interest in future studies to investigate the
400 ecotoxicological effects of dissolved Ce^{3+} in the presence of PAA and other common
401 stabilising agents.
402
403 In most studies with pristine CeO_2 ENPs stable dispersions in algal exposure media did not
404 form, with the ENPs undergoing different degrees of agglomeration (van Hoecke et al., 2009;
405 Rogers et al., 2010; Manier et al., 2011; Rodea-Palomares et al., 2011; Manier et al., 2013).
406 However, primary particle size was found to influence toxicity irrespective of agglomeration,
407 with smaller nominal diameters increasing growth inhibition (van Hoecke et al., 2009). The
408 CeO_2 particles used in the current study have a nominal diameter of 4-10 nm, whilst those
409 used in other studies with *P. subcapitata* are in the range 10-60 nm (van Hoecke et al., 2009;
410 Rogers et al., 2010; Manier et al., 2011; Rodea-Palomares et al., 2011; Manier et al., 2013).
411 Therefore, it is possible that the smaller diameter of the CeO_2 ENPs used in this study may be
412 contributing to the observed increase in toxicity compared to other studies. However, the
413 increased dispersion stability and lack of significant aggregation in the PAA-CeO_2 exposure
414 samples cannot be ruled out as a contributing factor to the higher toxicity observed in this
415 study compared to previous studies with pristine CeO_2 ENPs.
416
417 *3.5. Biokinetics*
418 In both the current study and other literature studies, it is unclear if the mechanism of toxicity
419 for CeO_2 nanoparticles to *P. subcapitata* is through uptake or by physical interaction of algal
420 cells with the particles. No evidence of algal flocculation in the presence of PAA-CeO_2 was

observed during the current study. HR-ICP-MS analysis of the total Ce concentration (dissolved and particulate) in the exposure media before and after removal of the algae suggest that during the 72 h exposure up to 38% of the total Ce becomes directly associated with the algal cells (Figure 3). However, it is unclear whether this association is direct uptake or adsorption of the PAA-CeO_2 onto the surface of the algal cells. In their study, Rodea-Palomares et al. (Rodea-Palomares et al., 2011) found no evidence of CeO_2 ENP uptake by cells, but that their toxic mode of action appeared to require direct contact between ENPs and cells. The authors suggest that cell damage most probably took place by cell wall and membrane disruption, possibly due to the oxidative activity of ceria. CeO_2 ENPs have been shown to induce flocculation and a clustering of particles on the cell surface of *P. subcapitata*, whereby the interaction of CeO_2 ENP with the cell surface also lead to an increase of cell membrane permeability (van Hoecke et al., 2009; Rodea-Palomares et al., 2011). However, when Röhder et al. (Röhder et al., 2014) compared a cell wall free mutant and a wild strain of the freshwater alga *Chlamydomonas reinhardtii* they concluded that cell wall plays a minor role on the toxicity to CeO_2 ENPs. Furthermore, a flocculation of cells was observed following exposure to agglomerated CeO_2 ENPs, and may represent a general response to various stresses (Rakesh et al., 2014), although whether this process impairs growth through shading or by limiting the diffusion of nutrients remains to be evaluated (Röhder et al., 2014).

Algal cells collected from the ecotoxicity experiments were analysed by TOF-SIMS in order to investigate their interaction with the PAA-CeO_2. Specific alterations in the cell membrane composition (see Figure 5) of *P. subcapitata* could be used to separate unexposed control cells at 0 h from unexposed control cells harvested after 72 h, and from PAA-CeO_2 exposed cells at 0 h and 72 h, using algal cell wall biomarker compounds (see Figure 4). Generally three membrane alterations can be identified (Figure 5 A-C). The first is an increase in certain

446 biomarker compounds directly after PAA-CeO_2 exposure (0 h) in comparison to unexposed
447 controls at both 0 h and 72h, indicating a direct response to the presence of the PAA-CeO_2
448 (Figure 5A). In particular, the acquired surface spectra of PAA-CeO_2 exposed algae at 0 h and
449 72h exposure showed a significant increase in ion m/e 327 (Yang et al., 2013), which is
450 tentatively assigned to a hydroxy eicosanoic acid. This hydroxy fatty acid is commonly found
451 in algae and micro algae (Blokker et al., 1998; Sasso et al., 2012), and in this study already
452 exhibited a significant increase at 0 h in PAA-CeO_2 exposed cells. Oxidised fatty acids are
453 known to be protective against oxidative stress and may even serve as signalling molecules
454 for inter-individual as well as inter-species communication (Pohl et al., 2014). A similar
455 behaviour is observed for ions m/e 504, m/e 846 and m/e 1600. Ion m/e 504 is tentatively
456 assigned to a lyso phosphatidyl ethanolamine (lyso PE C20:2). Lyso phosphatidyl
457 ethanolamines have already been identified in micro algae (He et al., 2011) and higher
458 amounts of lyso phosphatidyl ethanolamines are associated with the inhibition of
459 phospholipase D which causes enhanced cell wall lipid degradation and oxidative stress
460 (Munnik, 2001; Peters et al., 2007). Ion m/e 846 was tentatively assigned to a triacylglyceride
461 (TG C52:8). An increase in triacylglyceride levels was also observed in micro algae under
462 environmental stress and especially as a result of heavy metal exposure (Sharma et al., 2012).
463 Ion m/e 1600 could not be assigned to any known compound. These results are consistent
464 with reports describing the generation of reactive oxygen species (ROS) from CeO_2 ENPs
465 which are involved in CeO_2 ENP toxicity to mammalian cells (Auffan et al., 2009b). In
466 contrast, other studies have reported CeO_2 ENPs exhibit a scavenging ability and can reduce
467 oxidative stress (Amin et al., 2011). The contradictory ability of CeO_2 ENPs to both generate
468 and scavenge ROS seems to depend on the redox state, which can change between Ce(III) and
469 Ce(IV) (Auffan et al., 2009a). In the current study, PAA-CeO_2 appears to generate ROS
470 which elicit a response from the algal cells and maybe also be contributing to the observed

acute toxicity (growth inhibition). However, we are unable to conclude whether this represents high ROS formation compared to other CeO_2 ENPs studied and therefore contributing to the increased toxicity of the PAA-CeO_2. Furthermore, it is possible that the physicochemical properties of the CeO_2 ENPs and/or the presence of PAA is resulting in the increased formation of ROS which, in turn, may increase the observed toxicity.

A second alteration could be observed, where the control sample at 0 h had high levels of certain biomarker compounds, which were observed to be significantly decreased in 72 h controls and in both 0 h and 72 h PAA-CeO_2 exposed cells (Figure 5B). Control and PAA-CeO_2 exposed cells at 72 h exhibited slightly lower levels than PAA-CeO_2 exposed cells at 0 h. Ions m/e 341 and m/e 343 could be tentatively assigned to caffeic acid-O-glycoside and homovanillic acid-O-glycoside respectively. Micro algae have the capability to synthesize caffeic acid from the amino acid phenylalanine (El-Baky et al., 2009). This study also showed that caffeic acid exhibited antioxidant effects on CCI_4-induced lipid peroxidation and could serve as a radical scavenger in micro algae. The decrease in caffeic acid biosynthesis in the present study may indicate an age related loss of lipid peroxidation recovery and radical scavenging activity, which could be triggered already at time 0h in PAA-CeO_2 exposed cells. Ions m/e 895 and m/e 897, tentatively assigned to phosphatidylinositols (PI(O-C40:6) and PI(O-C40:5)) showed a similar mechanism.

A third biomarker alteration exhibited highest levels in both the unexposed and the exposed control cells at time point 0 h. Over the 72 h duration of the test, a significant decrease in the concentration of these compounds was observed in both sample types (Figure 5C). A number of previous studies have also reported a rapid change (e.g. within 5 mins) in the state of the cell membrane following exposure to oxidative stress causing chemicals (Alvarez-Ordóñez et

al., 2010; Hale et al., 2011). This mechanism always seems to be associated with a subsequent secondary change of the cell membrane, typically observed after 1 to 3 days. For the first time, the current study indicates specific biomarker compounds related to these mechanisms. The concentration of ions m/e 330 and m/e 332, tentatively assigned to sialic acid and dehydrosialic acid decreased in samples (both control and PAA-CeO_2 exposed) from 0 h to 72 h in a similar way. This indicates a general metabolic mechanism, unrelated PAA-CeO_2 exposure, is occurring in *P. subcapitata* cultures over time. Sialic acid probably arises from terminally sialylated N-linked oligosaccharides, which were already identified in green algae (Mamedov et al., 2011). Sialic acid decrease has already been characterized as a biomarker for muscle aging in mice and may also represent a biomarker for aging effects in *P. subcapitata* (Hanisch et al., 2013). The data show that this response in the algae is time dependent and not dependent upon PAA-CeO_2 exposure, representing basic age related metabolomic and lipidomic changes under conditions applied in the present study.

4. Conclusions

Under typical environmental conditions it is likely that PAA-stabilised CeO_2 ENPs will not undergo significant agglomeration and settle out of the aqueous phase. The use of stabilising agents in the synthesis of ENPs to provide useful physicochemical properties for technology applications may therefore lead to significant differences in the environmental behaviour compared to pristine ENP analogues. Stably dispersed PAA-CeO_2 appear to elicit a toxicological response in *P. subcapitata* at lower concentrations than pristine CeO_2 ENPs which rapidly agglomerate. Despite this, the PAA-CeO_2 concentrations needed to cause short-term effects appear to be much higher in comparison to the background cerium concentration in natural waters (Röhder et al., 2014). However, release of PAA-CeO_2 would offer the

possibility of increasing environmental concentrations of stably dispersed nanoparticle ceria in natural waters. Owing to the low dissolution rate of Ce^{3+}/Ce^{4+}, PAA-CeO_2 may have a considerable residence time in natural waters. As the modification of ENP surface chemistry and the use of stabilising agents is becoming more common in the synthesis of ENPs for technology applications, there is a need to generate new ecotoxicity data in addition to that available for 'pristine' materials.

Acknowledgements

The work reported here has been undertaken as part of the AERTO project 'Value from Waste', the EU FP7 project 'NANoREG' (Grant Agreement number 310584) and the Research Council of Norway project 'NanoSorb' (Grant Agreement number 209685/E50). The authors wish to thank these projects for their financial support. The authors acknowledge the essential technical assistance of Kristin Bonaunet, Lisbet Støen, Inger Steinsvik, Anne Rein Hatleveit, Calin Marioara (SINTEF Materials and Chemistry), Galina Alvarez (SP) and Syverin Lierhagen (NTNU).

References

Adegboyega NF, Sharma VK, Siskova K, Zbořil R, Sohn M, Schultz BJ, Banerjee S. Interactions of Aqueous Ag+ with Fulvic Acids: Mechanisms of Silver Nanoparticle Formation and Investigation of Stability. Environmental Science & Technology 2012; 47: 757-764.

Alvarez-Ordóñez A, Prieto M. Changes in Ultrastructure and Fourier Transform Infrared Spectrum of Salmonella enterica Serovar Typhimurium Cells after Exposure to Stress Conditions. Applied and Environmental Microbiology 2010; 76: 7598-7607.

Amin KA, Hassan MS, Awad el ST, Hashem KS. The protective effects of cerium oxide nanoparticles against hepatic oxidative damage induced by monocrotaline. Int J Nanomedicine 2011; 6: 143-149.

Auffan M, Rose J, Wiesner MR, Bottero J-Y. Chemical stability of metallic nanoparticles: A parameter controlling their potential cellular toxicity in vitro. Environmental Pollution 2009a; 157: 1127-1133.

Auffan M, Rose J, Orsiere T, De Meo M, Thill A, Zeyons O, Proux O, Masion A, Chaurand P, Spalla O, Botta A, Wiesner MR, Bottero J-Y. CeO2 nanoparticles induce DNA damage towards human dermal fibroblasts in vitro. Nanotoxicology 2009b; 3: 161-171.

Baalousha M, Nur Y, Römer I, Tejamaya M, Lead JR. Effect of monovalent and divalent cations, anions and fulvic acid on aggregation of citrate-coated silver nanoparticles. Science of the Total Environment 2013; 454–455: 119-131.

Blokker P, Schouten S, van den Ende H, De Leeuw JW, Sinninghe Damsté JS. Cell wall-specific ω-hydroxy fatty acids in some freshwater green microalgae. Phytochemistry 1998; 49: 691-695.

Booth A, Justynska J, Kubowicz S, Johnsen H, Frenzel M. Influence of salinity, dissolved organic carbon and particle chemistry on the aggregation behaviour of methacrylate-based polymeric nanoparticles in aqueous environments. International Journal of Environment and Pollution 2013; 52: 15 - 31.

Chane-Ching JY. Alkali metal and ammonium hydroxy nitrates with cerium. In: Google Patents, 1994.

El-Baky HHA, El Baz FK, El-Baroty GS. Production of phenolic compounds from Spirulina maxima microalgae and its protective effects. African Journal of Biotechnology 2009; 8: 7059-7067.

Gallego-Urrea JA, Perez Holmberg J, Hassellov M. Influence of different types of natural organic matter on titania nanoparticle stability: effects of counter ion concentration and pH. Environmental Science: Nano 2014; 1: 181-189.

García A, Espinosa R, Delgado L, Casals E, González E, Puntes V, Barata C, Font X, Sánchez A. Acute toxicity of cerium oxide, titanium oxide and iron oxide nanoparticles using standardized tests. Desalination 2011; 269: 136-141.

Haase A, Arlinghaus HF, Tentschert J, Jungnickel H, Graf P, Mantion A, Draude F, Galla S, Plendl J, Goetz ME, Masic A, Meier W, Thünemann AF, Taubert A, Luch A. Application of Laser Postionization Secondary Neutral Mass Spectrometry/Time-of-Flight Secondary Ion Mass Spectrometry in Nanotoxicology: Visualization of Nanosilver in Human Macrophages and Cellular Responses. ACS Nano 2011; 5: 3059-3068.

Hale John P, Winlove CP, Petrov Peter G. Effect of Hydroperoxides on Red Blood Cell Membrane Mechanical Properties. Biophysical Journal 2011; 101: 1921-1929.

581 Hanisch F, Weidemann W, Großmann M, Joshi PR, Holzhausen H-J, Stoltenburg G, Weis J,
582 Zierz S, Horstkorte R. Sialylation and Muscle Performance: Sialic Acid Is a Marker of
583 Muscle Ageing. PloS one 2013; 8: e80520.
584 He H, Rodgers RP, Marshall AG, Hsu CS. Algae Polar Lipids Characterized by Online Liquid
585 Chromatography Coupled with Hybrid Linear Quadrupole Ion Trap/Fourier Transform
586 Ion Cyclotron Resonance Mass Spectrometry. Energy & Fuels 2011; 25: 4770-4775.
587 Johnston BD, Scown TM, Moger J, Cumberland SA, Baalousha M, Linge K, van Aerle R, Jarvis
588 K, Lead JR, Tyler CR. Bioavailability of Nanoscale Metal Oxides TiO2, CeO2, and ZnO to
589 Fish. Environmental Science & Technology 2010; 44: 1144-1151.
590 Jungnickel H, Jones EA, Lockyer NP, Oliver SG, Stephens GM, Vickerman JC. Application of
591 TOF-SIMS with Chemometrics To Discriminate between Four Different Yeast Strains
592 from the Species Candida glabrata and Saccharomyces cerevisiae. Analytical
593 Chemistry 2005; 77: 1740-1745.
594 Karakoti AS, Monteiro-Riviere NA, Aggarwal R, Davis JP, Narayan RJ, Self WT, McGinnis J, Seal
595 S. Nanoceria as antioxidant: Synthesis and biomedical applications. JOM 2008; 60: 33-
596 37.
597 Keller AA, Wang H, Zhou D, Lenihan HS, Cherr G, Cardinale BJ, Miller R, Ji Z. Stability and
598 Aggregation of Metal Oxide Nanoparticles in Natural Aqueous Matrices.
599 Environmental Science & Technology 2010; 44: 1962-1967.
600 Loosli F, Le Coustumer P, Stoll S. TiO2 nanoparticles aggregation and disaggregation in
601 presence of alginate and Suwannee River humic acids. pH and concentration effects
602 on nanoparticle stability. Water Research 2013; 47: 6052-6063.
603 Louie SM, Tilton RD, Lowry GV. Effects of Molecular Weight Distribution and Chemical
604 Properties of Natural Organic Matter on Gold Nanoparticle Aggregation.
605 Environmental Science & Technology 2013; 47: 4245-4254.
606 Mamedov T, Yusibov V. Green algae Chlamydomonas reinhardtii possess endogenous
607 sialylated N-glycans. FEBS Open Bio 2011; 1: 15-22.
608 Manier N, Garaud M, Delalain P, Aguerre-Chariol O, Pandard P. Behaviour of ceria
609 nanoparticles in standardized test media – influence on the results of
610 ecotoxicological tests. Journal of Physics: Conference Series 2011; 304: 012058.
611 Manier N, Bado-Nilles A, Delalain P, Aguerre-Chariol O, Pandard P. Ecotoxicity of non-aged
612 and aged CeO2 nanomaterials towards freshwater microalgae. Environmental
613 Pollution 2013; 180: 63-70.
614 Munnik T. Phosphatidic acid: an emerging plant lipid second messenger. Trends Plant Sci
615 2001; 6: 227-233.
616 OECD. Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Proposal for updating
617 Guideline 201. Organisation for Economic Cooperation and Development (OECD),
618 Paris. 2002, 21.
619 OECD. Freshwater Alga and Cyanobacteria, Growth Inhibition Test. . Organisation for
620 Economic Cooperation and Development (OECD), Paris. 2011, 25.
621 Park B, Martin P, Harris C, Guest R, Whittingham A, Jenkinson P, Handley J. Initial in vitro
622 screening approach to investigate the potential health and environmental hazards of
623 EnviroxTM - a nanoparticulate cerium oxide diesel fuel additive. Particle and Fibre
624 Toxicology 2007; 4: 12.

625 Peters NT, Logan KO, Miller AC, Kropf DL. Phospholipase D signaling regulates microtubule
626 organization in the fucoid alga Silvetia compressa. Plant Cell Physiol 2007; 48: 1764-
627 1774.
628 Pohl C, Kock J. Oxidized Fatty Acids as Inter-Kingdom Signaling Molecules. Molecules 2014;
629 19: 1273-1285.
630 Quik JTK, Stuart MC, Wouterse M, Peijnenburg W, Hendriks AJ, van de Meent D. Natural
631 colloids are the dominant factor in the sedimentation of nanoparticles.
632 Environmental Toxicology and Chemistry 2012; 31: 1019-1022.
633 Quik JTK, Lynch I, Hoecke KV, Miermans CJH, Schamphelaere KACD, Janssen CR, Dawson KA,
634 Stuart MAC, Meent DVD. Effect of natural organic matter on cerium dioxide
635 nanoparticles settling in model fresh water. Chemosphere 2010; 81: 711-715.
636 Rakesh S, Saxena S, Dhar D, Prasanna R, Saxena A. Comparative evaluation of inorganic and
637 organic amendments for their flocculation efficiency of selected microalgae. J Appl
638 Phycol 2014; 26: 399-406.
639 Rodea-Palomares I, Boltes K, Fernández-Piñas F, Leganés F, García-Calvo E, Santiago J, Rosal
640 R. Physicochemical Characterization and Ecotoxicological Assessment of CeO2
641 Nanoparticles Using Two Aquatic Microorganisms. Toxicological Sciences 2011; 119:
642 135-145.
643 Rodea-Palomares I, Gonzalo S, Santiago-Morales J, Leganés F, García-Calvo E, Rosal R,
644 Fernández-Piñas F. An insight into the mechanisms of nanoceria toxicity in aquatic
645 photosynthetic organisms. Aquatic Toxicology 2012; 122–123: 133-143.
646 Rogers NJ, Franklin NM, Apte SC, Batley GE, Angel BM, Lead JR, Baalousha M. Physico-
647 chemical behaviour and algal toxicity of nanoparticulate CeO2 in freshwater.
648 Environmental Chemistry 2010; 7: 50-60.
649 Röhder LA, Brandt T, Sigg L, Behra R. Influence of agglomeration of cerium oxide
650 nanoparticles and speciation of cerium(III) on short term effects to the green algae
651 Chlamydomonas reinhardtii. Aquatic Toxicology 2014; 152: 121-130.
652 Salazar-Sandoval EJ, Johansson MKG, Ahniyaz A. Aminopolycarboxylic acids as a versatile
653 tool to stabilize ceria nanoparticles - a fundamental model experimentally
654 demonstrated. RSC Advances 2014; 4: 9048-9055.
655 Sánchez A, Recillas S, Font X, Casals E, González E, Puntes V. Ecotoxicity of, and remediation
656 with, engineered inorganic nanoparticles in the environment. TrAC Trends in
657 Analytical Chemistry 2011; 30: 507-516.
658 Sasso S, Pohnert G, Lohr M, Mittag M, Hertweck C. Microalgae in the postgenomic era: a
659 blooming reservoir for new natural products. FEMS Microbiology Reviews 2012; 36:
660 761-785.
661 Sehgal A, Lalatonne Y, Berret JF, Morvan M. Precipitation−Redispersion of Cerium Oxide
662 Nanoparticles with Poly(acrylic acid): Toward Stable Dispersions. Langmuir 2005; 21:
663 9359-9364.
664 Sharma KK, Schuhmann H, Schenk PM. High Lipid Induction in Microalgae for Biodiesel
665 Production. Energies 2012; 5: 1532-1553.
666 Tantra R, Schulze P, Quincey P. Effect of nanoparticle concentration on zeta-potential
667 measurement results and reproducibility. Particuology 2010; 8: 279-285.
668 Tentschert J, Draude F, Jungnickel H, Haase A, Mantion A, Galla S, Thünemann AF, Taubert A,
669 Luch A, Arlinghaus HF. TOF-SIMS analysis of cell membrane changes in functional

impaired human macrophages upon nanosilver treatment. Surface and Interface Analysis 2013; 45: 483-485.

Thompson CE, Jungnickel H, Lockyer NP, Stephens GM, Vickerman JC. ToF-SIMS studies as a tool to discriminate between spores and vegetative cells of bacteria. Applied Surface Science 2004; 231–232: 420-423.

US EPA. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. EPA-821-R-02-012. U.S. Environmental Protection Agency, Washington, DC. 2002, 31-33.

van Hoecke K, De Schamphelaere KAC, Van der Meeren P, Smagghe G, Janssen CR. Aggregation and ecotoxicity of CeO2 nanoparticles in synthetic and natural waters with variable pH, organic matter concentration and ionic strength. Environmental Pollution 2011; 159: 970-976.

van Hoecke K, Quik JTK, Mankiewicz-Boczek J, de Schamphelaere KAC, Elsaesser A, van der Meeren P, Barnes C, McKerr G, Howard CV, van de Meent D, Rydzyński K, Dawson KA, Salvati A, Lesniak A, Lynch I, Silversmit G, de Samber B, Vincze L, Janssen CR. Fate and Effects of CeO2 Nanoparticles in Aquatic Ecotoxicity Tests. Environmental Science & Technology 2009; 43: 4537-4546.

Wang H, Keller AA, Clark KK. Natural organic matter removal by adsorption onto magnetic permanently confined micelle arrays. Journal of Hazardous Materials 2011; 194: 156-161.

Wang N, Hsu C, Zhu L, Tseng S, Hsu J-P. Influence of metal oxide nanoparticles concentration on their zeta potential. Journal of Colloid and Interface Science 2013; 407: 22-28.

Xu C, Qu X. Cerium oxide nanoparticle: a remarkably versatile rare earth nanomaterial for biological applications. NPG Asia Mater 2014; 6: e90.

Yang N-Y, Yang Y-F, Li K. Analysis of Hydroxy Fatty Acids from the Pollen of Brassica campestris L. var. oleifera DC. by UPLC-MS/MS. Journal of Pharmaceutics 2013; 2013: 6.

Figures

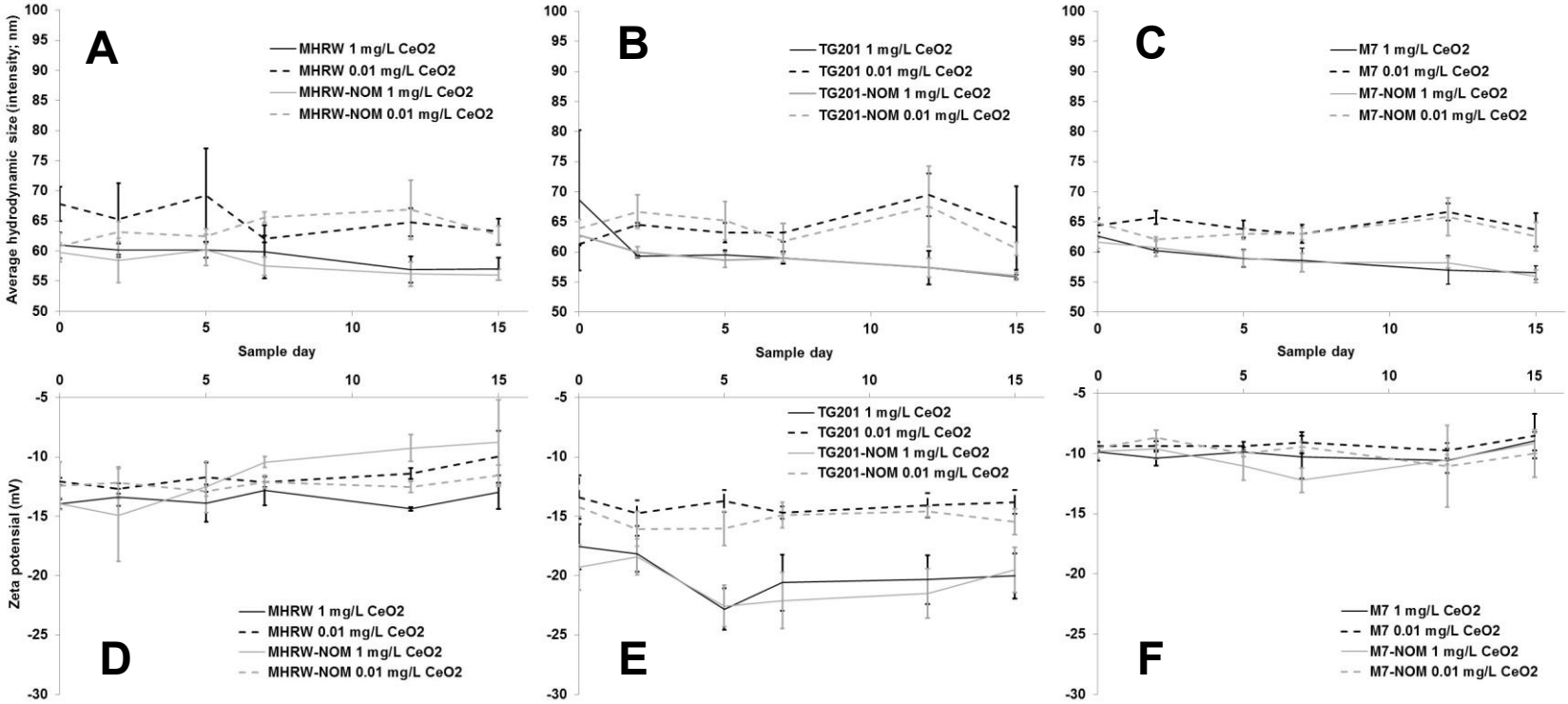
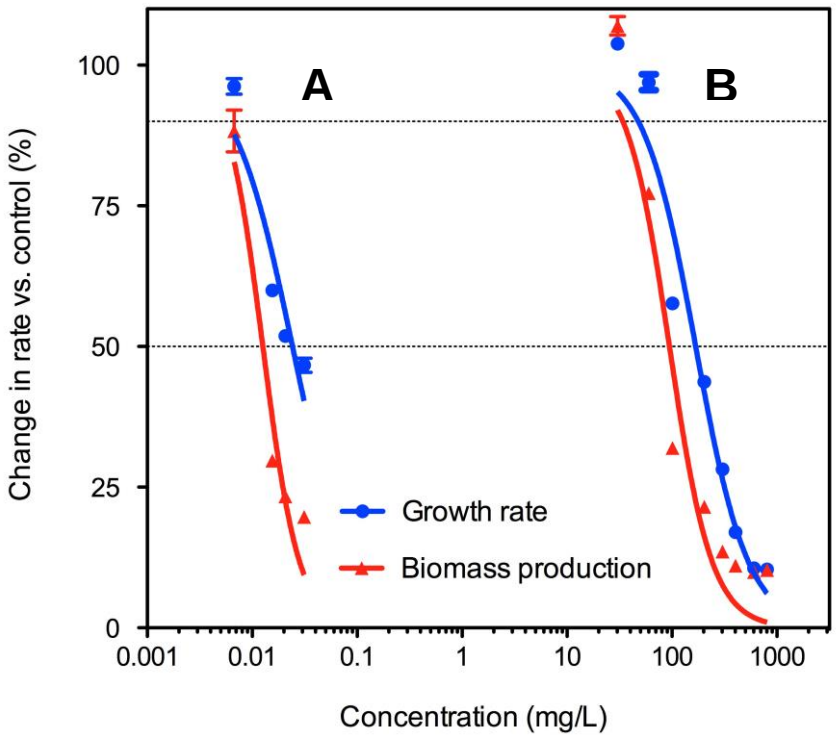
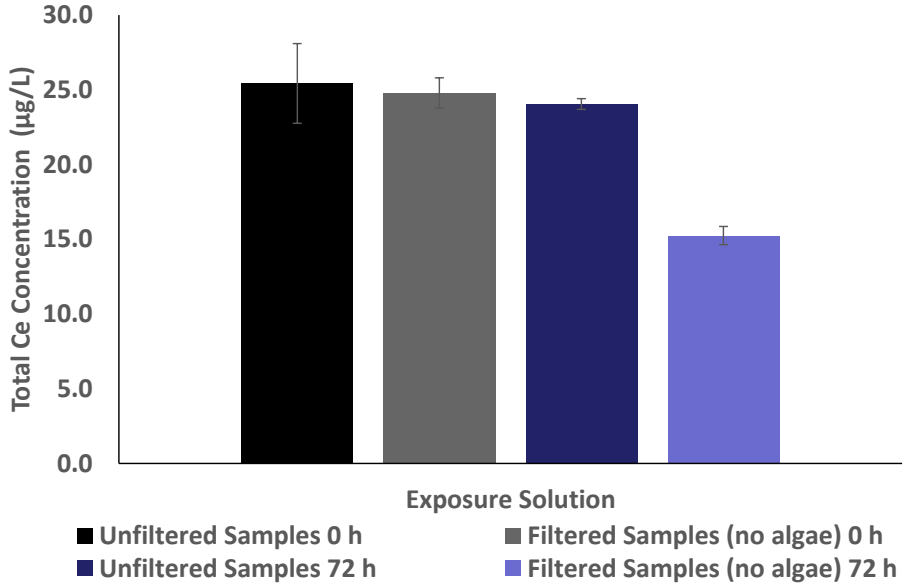


Figure 1. Hydrodynamic size (nm) in A) MHRW, B) TG201 and C) M7 media and zeta potential (mV) measurements in D) MHRW, E) TG201 and F) M7 media during 15 day stability studies at two different suspension concentrations of PAA-CeO_2 (0.01 and 1.0 mg/L). Hydrodynamic size is displayed as intensity averaged sizes. Error bars represent standard deviation (n=3).



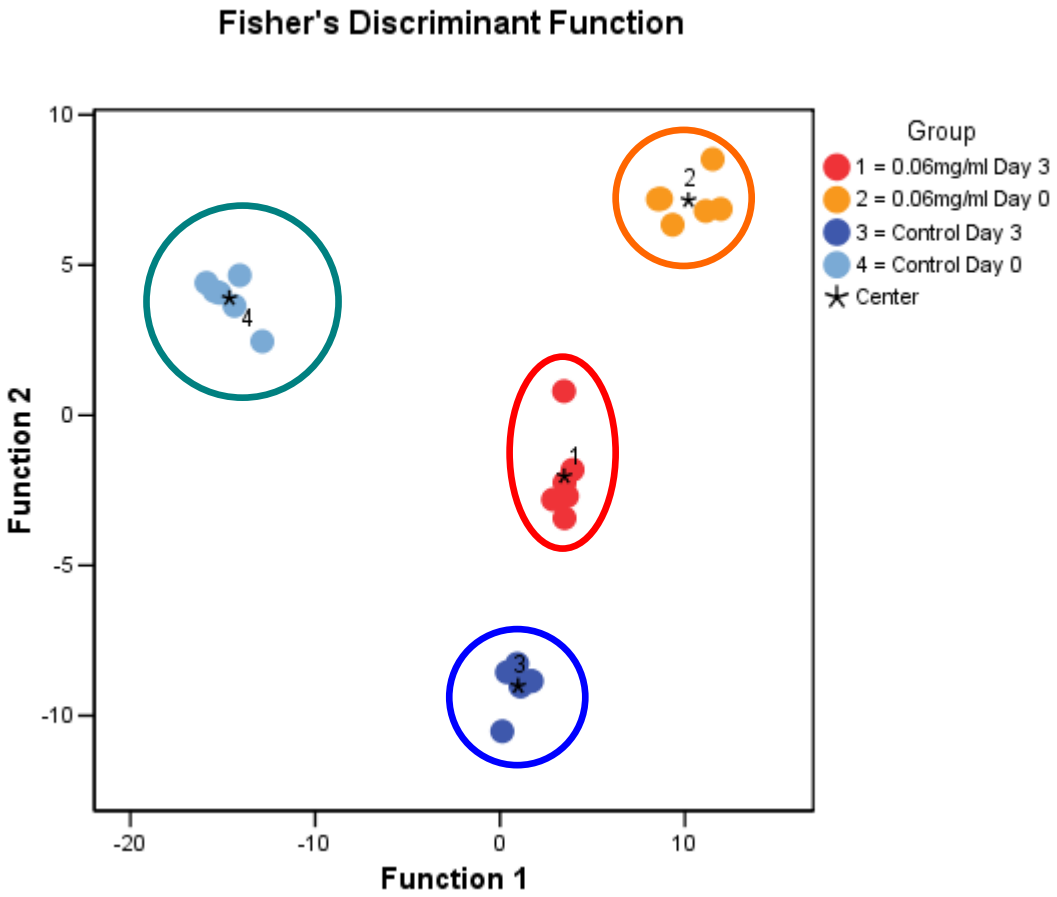
705

706 *Figure 2. Change in growth rate and biomass production as a function of A) PAA-CeO_2 and*
707 *B) pure PAA. Both data sets are plotted according to the CeO_2 exposure concentrations*
708 *determined using HR-ICP-MS.*
709



710

711 *Figure 3. Total Ce concentration (µg/L) in filtered samples (no algae) and unfiltered samples*
712 *(containing algae) collected at 0 h and 72 h. Error bars represent standard deviation (n=3).*
713



714

715

716 *Figure 4. TOF-SIMS analysis changes in compound composition of the cell wall of*
717 *Pseudokirchneriella subcapitata after nanoparticle treatment. The diagram shows the values*
718 *of the discriminant scores obtained from Fisher's discriminant analysis of 24 algal samples*
719 *for all ions, which were selected to discriminate between untreated micro algae cultures at*
720 *day 0 and day 3 and micro algae, treated with 0.06mg/ml CeO_2 at day 0 and day 3.*
721

722

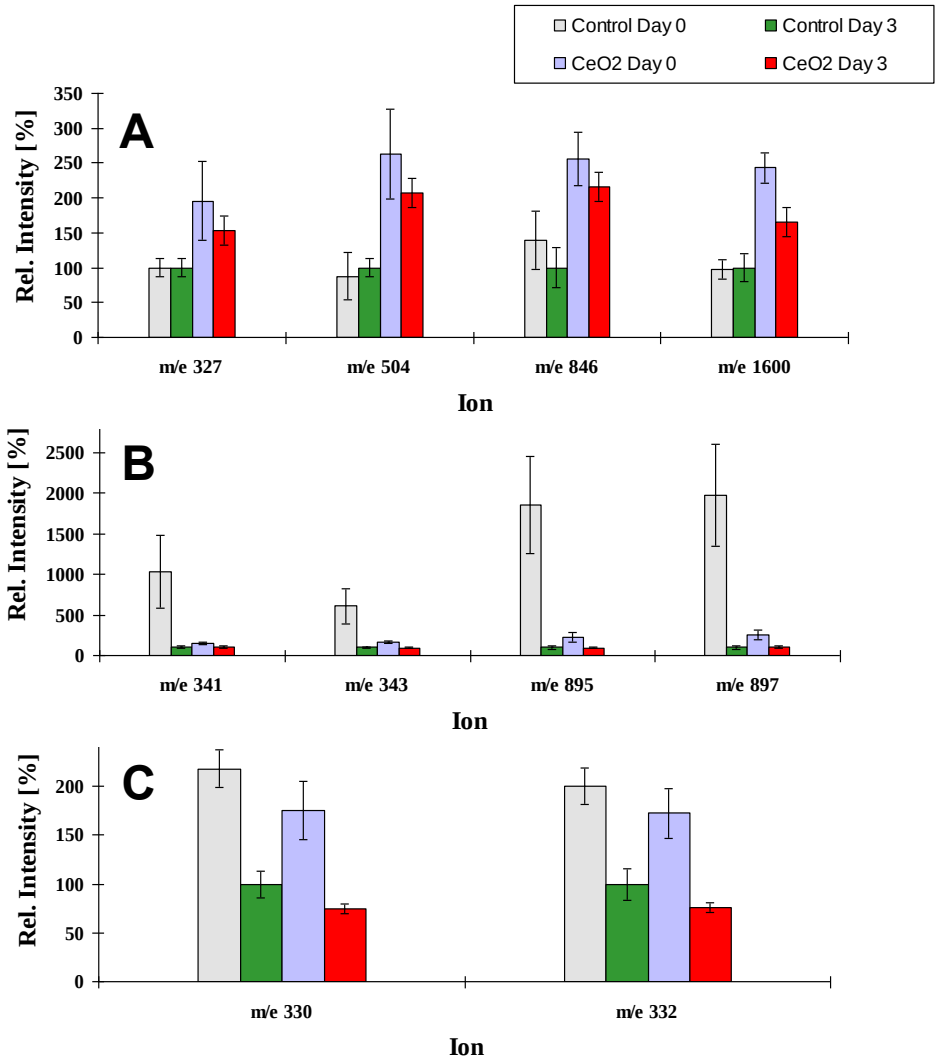


Figure 5. Histogram comparisons of ion yields for characteristic biomarker ions which were used to separate the four treatment groups. The biomarker ions indicate three different biomarker alterations: A) where compounds loaded high on function 1 (>0.9; Figure 4) and showed significantly higher levels in exposed samples at day 0 and day 3 in comparison to control samples. B) where compounds loaded high (>0.9; Figure 4) on function 1 or function 2 and showed significantly higher levels in control samples at day 0 compared to controls at day 3 and exposed cells at day 0 and day 3. C) where compounds loaded high (>0.9; Figure 4) on function 1 or function 2 and showed significantly higher levels in control and exposed samples at day 0 compared to control and exposed samples at day 3. For the relative intensity, the mean of the control group at 72 h was taken as 100% in all cases.

Tables

Table 1. Summary of the nominal PAA-CeO_2 concentrations, total Ce concentration at 0 h and 72 h, the calculated dissolved Ce concentration at 0 h and the free PAA concentration at 0 h in each of the exposure samples used in growth inhibition tests with P. subcapitata.

Nominal PAA-CeO_2 exposure concentration (µg/L)	**Total Ce concentration (µg/L)**				**Dissolved Ce concentration at 0 h (µg/L)**	**Free PAA concentration at 0 h (µg/L)**
	0 h	**%rsd**	**72 h**	**%rsd**		
15	5.5	2.8	6.5	2	0.5	9.6
25	12.6	8.8	10.2	1.5	1.0	16
40	16.8	4.4	15.3	1.8	1.4	25.6
60	25.4	10.5	24.0	1.5	2.1	38.4
100	32.7	9.5	52.4	4.4	2.7	64
200	67.5	12	81.0	4.5	5.6	128

Table 2. Calculated effect concentrations of pure PAA and PAA stabilised CeO_2 nanoparticles (PAA-CeO_2) to the freshwater algae, Pseudokirchneriella subcapitata*, in a 72 h growth inhibition test.*

	EC_{10} mg/L (95% CI)		**EC_{50} mg/L (95% CI)**	
	Growth Rate	*Biomass production*	*Growth Rate*	*Biomass production*
PAA	**47.7** (36.2 - 63.1)	**34.0** (24.3 - 47.6)	**168.5** (149.3 - 190.1)	**94.7** (81.3 - 110.3)
PAA-CeO_2	**0.0058** (0.0036 - 0.0094)	**0.0053** (0.0037 - 0.0074)	**0.024** (0.021 - 0.028)	**0.013** (0.011 - 0.015)