Interactions of cerium dioxide nanoparticles with the green alga *Chlamydomonas reinhardtii*: influence of physico-chemical characteristics and cerium(III)

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Content

Content................................................................................................................................................. I
Summary .................................................................................................................................................. IV
Zusammenfassung ..................................................................................................................................... VI
Chapter 1 .................................................................................................................................................. 1
  1.1 Introduction ........................................................................................................................................ 2
  1.2 Applications of CeO₂ NP .................................................................................................................. 2
  1.3 Physicochemical properties of CeO₂ NP ......................................................................................... 3
  1.4 Size measurement of nanoparticles .............................................................................................. 4
  1.5 Release of CeO₂ NP to the environment ......................................................................................... 5
  1.6 Fate of CeO₂ NP in the environment .............................................................................................. 6
  1.7 Effects of CeO₂ NP and cerium(III) to algae ................................................................................. 8
  1.8 Scope of the thesis .......................................................................................................................... 10
  1.9 References ....................................................................................................................................... 11
Chapter 2 .................................................................................................................................................. 17
  2.1 Abstract ........................................................................................................................................... 18
  2.2 Introduction ...................................................................................................................................... 19
  2.3 Materials and methods .................................................................................................................... 21
    2.3.1 Preparation of CeO₂ NP suspensions ......................................................................................... 21
    2.3.2 Characterization of CeO₂ NP .................................................................................................... 21
    2.3.3 Metal analysis ............................................................................................................................ 22
    2.3.4 Algae cultures ........................................................................................................................... 23
    2.3.5 Exposure media and conditions ............................................................................................... 23
    2.3.6 Photosynthesis ........................................................................................................................ 24
    2.3.7 Intracellular ROS level ............................................................................................................ 24
  2.4 Results ............................................................................................................................................. 26
    2.4.1 Stability of CeO₂ NP suspensions ............................................................................................ 26
    2.4.2 Effect on photosynthetic yield and intracellular ROS level ..................................................... 28
    2.4.3 Morphology .............................................................................................................................. 33
  2.5 Discussion ......................................................................................................................................... 35
    2.5.1 Behavior of CeO₂ NP in exposure media .................................................................................. 35
    2.5.2 Effect of CeO₂ NP and cerium(III) on photosynthetic yield and intracellular ROS level ....... 35
  2.6 References ......................................................................................................................................... 39
  2.7 Supporting information .................................................................................................................... 43
Chapter 5 ................................................................. 95

5.1 Effects of CeO$_2$ NP ........................................... 96
5.2 Effects of cerium(III) ......................................... 97
5.3 Effects in natural aquatic systems ....................... 97
5.4 References ....................................................... 99

Acknowledgements .................................................. 100

Curriculum vitae .................................................... 101
Summary

Cerium oxide nanoparticles (CeO₂ NP) are increasingly used in industrial applications and are expected to be released into the aquatic environment. With their release into the environment an exposure of organisms becomes likely. Thus effects of CeO₂ NP to organisms have to be carefully evaluated to be able to assess risks. In this study the behavior of CeO₂ NP in algae growth medium was examined. Further the effects to the green algae *Chlamydomonas reinhardtii* as a model organism, were investigated in short and long term exposures. To discriminate between effects from CeO₂ NP and dissolved cerium(III) co-occurring in CeO₂ NP suspensions, experiments were also performed with Ce(NO₃)₃. The role of the cell wall of *C. reinhardtii* as a barrier for uptake and its influence on the sensitivity was evaluated by testing both, the wild type and the cell wall free mutant of *C. reinhardtii*.

Strong agglomeration of CeO₂ NP was observed in algae media at physiological pH. With the addition of phosphate CeO₂ NP were stabilized at pH 7.5 in exposure media. This effect was exploited to test CeO₂ NP dispersed with phosphate with a mean size of 140 nm and agglomerated in absence of phosphate. The level of dissolved cerium(III) in CeO₂ NP suspensions in all tested media was very low and between 0.1-27 nM. The effects of CeO₂ NP to *C. reinhardtii* and the role of dissolved cerium(III) on toxicity was assessed by measuring photosynthetic yield and intracellular reactive oxygen species (ROS) upon short term exposure. The photosynthetic yield of *C. reinhardtii* decreased as function of cerium(III) with EC₅₀ of 7.5 ± 0.84 µM for wild type and EC₅₀ of 6.3 ± 0.53 µM for the cell wall free mutant. The intracellular level of ROS increased upon exposure to cerium(III) but not upon exposure to CeO₂ NP. A slight decrease of photosynthetic yield was only measured upon exposure to the agglomerated CeO₂ NP at the highest concentrations (100 µM), while no effect was observed for dispersed CeO₂ NP. The low toxicity of agglomerated CeO₂ NP was attributed quantitatively to Ce³⁺ ions co-occurring in the nanoparticle suspension. In case of dispersed CeO₂ NP, dissolved Ce³⁺ was complexed with phosphate and thus not bioavailable. The sensitivity of the cell wall free mutant was comparable to that of the wild type. For both algae strains, a strong floculation of cells was observed upon exposure to agglomerated CeO₂ NP.

Furthermore it was explored whether the short term exposure of CeO₂ NP and cerium(III) to *C. reinhardtii* results in uptake of cerium. Wash steps with fresh medium containing EDTA
were done to remove CeO$_2$ NP and Ce$^{3+}$ from algal surfaces. A concentration and time dependent increase of cellular CeO$_2$ NP was measured. Based on the calculated number of CeO$_2$ NP per cell, an internalization of CeO$_2$ NP in C. reinhardtii was excluded. The increase of cellular cerium was explained by a sorption of CeO$_2$ NP to the cell wall. For cerium(III) maximal cellular cerium concentrations of 6.04 x 10$^{-4}$ mol L$_{cell}^{-1}$ in the wild type and 9.0 x 10$^{-5}$ mol L$_{cell}^{-1}$ in the cell wall free mutant of C. reinhardtii were measured after two hours of exposure. The increment of cellular cerium over time was in relation with the decrease of photosynthetic yield, which was similar in both algae strains. Competition experiments with calcium decreased the cellular cerium concentration in the wild type threefold, but toxicity did not decrease. In the cell wall free mutant cellular cerium concentrations and toxicity did not change in presence of calcium. The results indicated that cerium, which appeared as intracellular, was partly bound to the cell wall of the wild type. The slow uptake of cerium(III) indicated that no efficient transport routes are available in C. reinhardtii.

The effects of CeO$_2$ NP on the wild type and cell wall free mutant of C. reinhardtii were assessed in long term exposure over five days. Exponential growth of algae was maintained during exposure by exchanging the growth media and diluting the cell density every day. Growth, cell volume, photosynthetic yield and ATP content were measured at different time points during exposure. Controls and algae exposed to 10 µM agglomerated CeO$_2$ NP showed exponential growth in all subcultures. Exposed algae of both strains did not differ from the control in cell volume, photosynthetic yield and ATP content. No harmful effects of CeO$_2$ NP to the wild type and cell wall free mutant were observed in long term exposure.

In this work no direct effects of CeO$_2$ NP to the green alga C. reinhardtii were detected. The toxicity of CeO$_2$ NP was related to indirect effects of dissolved cerium(III). Moreover CeO$_2$ NP with a mean size of 140 nm were not internalized by algae. Cerium(III) was shown to be toxic to C. reinhardtii and to be taken up into the cells. Considering the detected effects of CeO$_2$ NP, the EC$_{50}$ values of cerium(III) and the predicted environmental concentration of CeO$_2$ NP, no effects on algae are expected to occur under environmental conditions.
Zusammenfassung

Ceroxid Nanopartikel (CeO₂ NP) kommen zunehmend in industriellen Anwendungen zum Einsatz und können so in die aquatische Umwelt gelangen. Mit ihrer Freisetzung in die Umwelt wird eine Exposition von Organismen erwartet. Um dadurch entstehende Risiken beurteilen zu können, ist es wichtig die Effekte von CeO₂ NP auf Organsimen genau zu untersuchen. In dieser Studie wurde das Verhalten von CeO₂ NP in Wachstumsmedium für Algen bestimmt und Effekte auf den Modelorganismus *Chlamydomonas reinhardtii* in Kurz- und Langzeitexpositionen untersucht. Um zwischen Effekten der CeO₂ NP und gelöstem Cer(III), welches in CeO₂ NP Suspensionen vorkommt, unterscheiden zu können, wurden zusätzlich Expositionen mit Ce(NO₃)₃ durchgeführt. Die Rolle der Zellwand von *C. reinhardtii* als Barriere für die Aufnahme und deren Einfluss auf die Sensitivität wurde mit einer zellwandfreien Mutante und dem Wildtyp von *C. reinhardtii* getestet.

Bei physiologischen pH wurde eine starke Agglomerationen der CeO₂ NP im Wachstumsmedium der Algen beobachtet. Durch Zugabe von Phosphat konnten CeO₂ NP im Medium bei einem pH von 7,5 stabilisiert werden. Dieser Effekt wurde ausgenutzt um dispergierte CeO₂ NP mit einer durchschnittlichen Größe von 140 nm in phosphathaltigem Medium und agglomerierte CeO₂ NP in phosphatfreiem Medium zu untersuchen. Die Menge an gelöst vorliegendem Cer(III) in den CeO₂ NP Suspensionen der verschiedenen Medien war sehr gering und lag zwischen 0.1 und 27 nM. Effekte der CeO₂ NP auf *C. reinhardtii* und die Rolle von gelöstem Cer(III) auf die Toxizität wurde durch die Messung der Photosyntheserate und dem intrazellulären Level von reaktiven Sauerstoffspezies (ROS) beurteilt. Hierbei verringerte sich die Photosyntheserate von *C. reinhardtii* mit steigender Cer(III) Konzentration, mit einem EC₅₀ von 7,5 ± 0,84 μM für den Wildtyp und 6,3 ± 0,53 μM für die zellwandfreien Mutante. Die Menge an intrazellulären ROS erhöhte sich in Folge der Exposition zu Cer(III), nicht aber in Folge der Exposition mit CeO₂ NP. Ein leichter Rückgang der Photosyntheserate erfolgte nur nach Exposition mit den höchsten Konzentrationen (>100 μM) von agglomerierten CeO₂ NP, während kein Effekt der dispergierten CeO₂ NP gefunden wurde. Die geringe Toxizität der agglomerierten CeO₂ NP konnte quantitativ auf die Konzentration an freien Ce³⁺ Ionen der phosphatfreien NP Suspension zurückgeführt werden. Im Gegensatz hierzu, waren in der dispergierten CeO₂ NP Suspension keine Ce³⁺ Ionen bioverfügbar, da sie durch Phosphat komplexiert wurden. Die zellwandfreie Mutante
wies eine vergleichbare Sensitivität zum Wildtyp auf. Für beide Algenstämme wurde während der Exposition mit agglomerierten CeO₂ NP eine starke Flockung der Algenzellen beobachtet.

Weiterhin wurde untersucht, ob die Kurzzeitexposition von C. reinhardtii mit CeO₂ NP und Cer(III) in einer Aufnahme von Cer resultiert. Es wurden Waschschritte mit frischem Medium welches EDTA enthielt durchgeführt, um CeO₂ NP und Ce³⁺ von der Algenoberfläche zu entfernen. Eine konzentrations- und zeitabhängige Zunahme der, an die Zellen gebundenen, CeO₂ NP wurde gemessen. Basierend auf der berechneten Anzahl von CeO₂ NP pro Zelle wurde einer Internalisierung von CeO₂ NP in C. reinhardtii ausgeschlossen, wobei der gemessene Anstieg der Cer Konzentration durch eine Sorption von CeO₂ NP an die Zellwand erklärt wurde. Nach zweistündiger Exposition mit Cer(III) wurde eine maximale zelluläre Cer Konzentration von 6,04 x 10⁻⁴ mol L⁻¹Zelle⁻¹ im Wildtyp und 9,0 x 10⁻⁵ mol L⁻¹Zelle⁻¹ in der zellwandfreien Mutante von C. reinhardtii gemessen. Die über die Expositionszeit ansteigende zelluläre Cer Konzentration stand im Zusammenhang mit der Abnahme der Photosyntheserate, welche in beiden Algenstämmen gleich war. Kompetitionsexperimente mit Calcium führten zu einer dreifach geringeren zellulären Cer Konzentration im Wildtyp, wobei die Toxizität unverändert blieb. In der zellwandfreien Mutante trat keine Veränderung der zellulären Cer Konzentration und der Toxizität unter Beigabe von Calcium auf. Dies deutete darauf hin, dass Cer, welches als zellulär vorliegend angenommen wurde, zum Teil an der Zellwand des Wildtyps gebunden vorlag. Die langsame Aufnahme von Cer lässt darauf schließen, dass keine effizienten Transportwege für Cer in C. reinhardtii vorhanden sind.

In dieser Arbeit konnten keine direkten Effekte der CeO$_2$ NP Exposition auf die Grünlage *C. reinhardtii* festgestellt werden. Die aufgetretene Toxizität der CeO$_2$ NP wurde gelöstem Cer(III) zugeschrieben. CeO$_2$ NP mit einer durchschnittlichen Größe von 140 nm wurden nicht von den Algen aufgenommen. Es konnte gezeigt werden, dass Cer(III) toxisch auf *C. reinhardtii* wirkte und in die Zellen aufgenommen wurde. In Anbetracht der gemessenen Effekte von CeO$_2$ NP, der EC$_{50}$-Werte von Cer(III) sowie den vorhergesagten Umweltkonzentration von CeO$_2$ NP, werden keine Auswirkungen der CeO$_2$ NP auf Algen unter Umweltbedingungen erwartet.
Chapter 1

General introduction
1.1 Introduction

Cerium is a rare earth element belonging to the lanthanide group. Commercial applications of cerium are numerous. The use of cerium oxide nanoparticles (CeO₂ NP) in a wide range of applications will certainly result in their release in the environment. Nowadays, environmental concentrations are unknown and can only be estimated. The fate of CeO₂ NP in the aquatic environment is currently unclear and only little data is available. A potential risk for the environment through harmful interactions with organisms has to be carefully evaluated and the assessment of ecotoxicological effects of CeO₂ NP is required. Thus, CeO₂ NP were included on the OECD list of priority nanomaterials for immediate testing (OECD, 2008).

In the present thesis the effects of CeO₂ NP and cerium(III) to the freshwater green alga Chlamydomonas reinhardtii were investigated in short and long term exposures. The physico-chemical characteristics of CeO₂ NP, such as agglomeration, dispersion and release of dissolved cerium(III) were carefully evaluated under exposure conditions.

1.2 Applications of CeO₂ NP

CeO₂ NP are utilised in a variety of applications ranging from emission reduction technology to therapeutics. The ability of CeO₂ NP to possibly fight chronic inflammation and pathologies associated with oxidative stress gives CeO₂ NP a pharmacological potential (Celardo et al., 2011). CeO₂ NP have the ability to absorb UV radiation, while being transparent to visible light and are therefore considered to be used in sunscreens (Yabe and Sato, 2003). The ability of absorbing UV radiation made CeO₂ NP also interesting for applications in coatings (e.g. wood paint) and despite the fact that CeO₂ NP are dispersed the coating formulations remain transparent (Ngoc Nhiem et al., 2011). Due to the intrinsic hardness, CeO₂ NP are an efficient glass polishing agent and used for glass mirrors, lenses and precision optics (Tsai, 2004). Furthermore cerium oxide is used as a glass constituent to prevent solarization and discoloration (Krogman et al., 2005).

CeO₂ NP play a key role in industrial catalysis due to the reversibility of its redox cycle, this property is regarded as oxygen storage capacity (Trofarelli et al., 1999). Therefore CeO₂ NP are utilized in oxygen gas sensor and fuel cells (Izu et al., 2004; Murray et al., 1999). Due to their oxidative capacity CeO₂ NP are applied as a diesel fuel additive. It was shown that CeO₂ NP reduce the fuel consumption, improve the combustion and reduce the soot in the
exhaust gas (Arul Mozhi Selvan et al., 2009; Jung et al., 2004; Saijith et al., 2010). Envirox is a trade name of a CeO₂ NP based diesel fuel catalyst and is used in the vehicle with a final CeO₂ NP concentration of 5 ppm. The efficiency of Envirox is mainly due to the vastly increased surface area of the nanoparticulate cerium oxide (Park et al., 2007).

### 1.3 Physicochemical properties of CeO₂ NP

While most of the rare earth elements exist in the trivalent state, cerium also occurs in the tetravalent state and can change between the two redox states (Korsvik et al., 2007). In the lattice structure of CeO₂ NP each cerium cation is coordinated by eight neighboring oxygen anions, which represents the fully oxidized form. Reduced cerium results from the removal of O²⁻ ions from the CeO₂ NP lattice, which generates an anion vacant site. The electrostatic balance is maintained by the reduction of two cerium cations from the tetravalent to the trivalent state at the particle surface (Perullini et al., 2013). Studies have shown that with a decrease in particle size, CeO₂ NP show a formation of more oxygen vacancies (Deshpande et al., 2005). The redox properties and the formation of oxygen vacancies play an important role for the application of CeO₂ NP and are of particular interest for the effect of CeO₂ NP on organisms. These reactions may on one hand lead to the formation of reactive oxygen species (ROS), and on the other hand to ROS scavenging reactions. CeO₂ NP have been shown to mimic both, superoxide dismutase and catalase activity. The superoxide dismutase activity of CeO₂ NP is the possible mechanism for the antioxidant properties. It is suggested that CeO₂ NP with a high Ce(III)/Ce(IV) ratio on the surface are able to reduce superoxide to peroxide with a high efficiency. Thereby Ce(III) is oxidized to Ce(IV) and hydrogen peroxide is generated (Korsvik et al., 2007). On the other hand Ce(IV) can be reduced to Ce(III) by the superoxide anion (O₂⁻).

\[
O_2^- + Ce(III) + 2H^+ \rightarrow Ce(IV) + H_2O_2
\]

\[
O_2^- + Ce(IV) \rightarrow O_2 + Ce(III)
\]

The restoration of reduced Ce(III) (Patil et al., 2007) can be mediated through H₂O₂, which can oxidize cerium present as Ce(III) to Ce(IV) (Heckert et al., 2008; Pirmohamed et al., 2010). Ce(IV) in turn can react with hydrogen peroxide resulting in water and oxygen and represents the catalase activity of CeO₂ NP. The catalase activity of CeO₂ NP is redox-state dependent and a high level of Ce(IV) leads to a rapid decrease of H₂O₂ in suspension (Pirmohamed et al., 2010).
The scavenging reactions of CeO₂ NP were shown to reduce ROS in different types of cells. It is proposed that the scavenging activity in vitro is related to the mixed valence states on the surface of CeO₂ NP (Das et al., 2007; Schubert et al., 2006). The reactivity of CeO₂ NP can be influenced by pH and the presence of phosphate and other anions by changing the ratio of Ce(III)/(IV) (Singh et al., 2011). Beside the scavenging reactions, CeO₂ NP are also involved in production of ROS, but the conditions under which ROS scavenging and ROS production occurs are unclear. In several toxicity experiments an increase of ROS and oxidative damage was found in cells upon contact to CeO₂ NP (Hirst et al., 2009; Horie et al., 2011; Park et al., 2008b).

The change of the oxidation state on the surface of CeO₂ NP can also influence the solubility of CeO₂ NP. Ce(IV) shows a very low solubility at physiological pH and becomes only soluble at low pH (pH <4) and would precipitate at higher pH. Reduction reactions on the CeO₂ NP surface can result in Ce(III), which solubility is higher over a wide pH range with the formation of Ce(OH)₃(s) or of Ce₂O₃(s) at pH >10 (Hayes et al., 2002; Yu et al., 2006). So far a low dissolution of CeO₂ NP was measured in different media with a level of dissolved Ce(III) below 0.5% (Rogers et al., 2010; Schwabe et al., 2013).

1.4 Size measurement of nanoparticles

Size measurement of nanoparticle suspensions can be performed with different techniques such as dynamic light scattering (DLS) and nanoparticle tracking analyses (NTA). Both methods are based on the detection of Brownian motion of particles in a liquid. Therefore particles are illuminated with a laser and the scattered light is detected. The relationship between the size of a particle and its speed due to Brownian motion is defined in the Stokes-Einstein equation. The size of the nanoparticles is measured as their hydrodynamic diameter. NTA allows nanoparticles to be analyzed based on a particle-by-particle visualization, whereas DLS measures the backscattered light of the entire suspension.

Both, NTA and DLS show a good sizing accuracy and relatively narrow distributions for all monodisperse samples. For DLS the size range for accurate measurements is between 1-1000 nm, and is more limited for NTA, which gives reproducible measurements between 20-800 nm (Röhder et al., 2011). While NTA requires a particle concentration of 10⁷-10⁹ particles/mL, the DLS concentration range is less critical and can be 10⁸-10¹² particles/mL.
(Filipe et al., 2010). In general the size distribution and mean size of particles measured by NTA is smaller as in DLS and is reported by different studies (Domingos et al., 2009; Kadar et al., 2010). An overestimation of particle size and a tailing of the DLS size distribution towards larger sizes is mostly due to the presence of a few larger particles in suspension (Biddle et al., 1996). As soon as polydisperse suspensions are measured with a smaller amount of small particles, NTA has clear advantages over DLS. NTA was shown to resolve and distinguish particle sizes in mixtures of two or more polystyrene standards, whereas DLS can only resolve particle populations that differ in size by a factor of three (Filipe et al., 2010; Röhder et al., 2011). NTA also provides information on concentration and this can be helpful for polydisperse suspensions (Gallego-Urrea et al., 2010). The different size measurement techniques can give complementary information on particle suspensions. In general, polydispersity of nanoparticle suspensions poses a major challenge for accurate size determination.

Microscopic techniques can be used to determine size, such as transmission electron microscopy (TEM) which allows a visualization of particles but requires a complex sample preparation. Other techniques can be used to determine the size of nanoparticles in suspension. These include single particle ICP-MS, flow field flow fractionation, atomic force microscopy and fluorescence correlation spectroscopy (Baalousha et al., 2012; Domingos et al., 2009; Scheffer et al., 2008).

### 1.5 Release of CeO$_2$ NP to the environment

The release of CeO$_2$ NP as single particles or as agglomerates into the environment has been barely studied. Quantitative data on the release of CeO$_2$ NP and environmental concentrations are rare. Much of the unintentional release of CeO$_2$ NP will be via wastewater or solid waste. Hence, wastewater treatment plants and incineration plants can be important sources for the distribution of CeO$_2$ NP (Gottschalk and Nowack, 2011). The release of CeO$_2$ NP from a model wastewater treatment plant was studied. A significant fraction of CeO$_2$ NP escaped the wastewater clearing system and up to 6% of CeO$_2$ NP was found in the exit stream (Limbach et al., 2008). In a different study an extensive removal of CeO$_2$ NP was attained in municipal wastewater treatment plant and a low concentration of 0.11 mg/L escaped the treatment (Gómez-Rivera et al., 2012). An introduction of CeO$_2$ NP into a solid-waste incineration plant showed that CeO$_2$ NP did not undergo physical or
chemical changes during combustion. CeO₂ NP mainly bound to residues from the combustion and were efficiently removed in the flue gas by the current filter technology (Walser et al., 2012). The release of CeO₂ NP can also occur by the use as a diesel fuel additive. Engine tests have shown, that CeO₂ NP fuel additive improved the combustion efficiency of diesel and showed as well that a small amount of cerium emitted in the exhaust gas was found to be in the nanoscale (Batley et al., 2013; Jung et al., 2004). Upon the introduction of CeO₂ NP as a diesel fuel additive the cerium concentration in particular dust (PM₁₀) was measured at different sites in the UK. Only at one monitoring site a significant increase of cerium was measured (Park et al., 2008a).

Increased air concentrations of CeO₂ NP can contribute to the presence of these particles in other environmental media, such as water and soil. Environmental concentrations in surface waters and soil of CeO₂ NP were predicted, based on the commercial information on the use of CeO₂ NP as fuel additive in the UK. For soils within 20 m of a road the highest predicted contamination level was 0.04 mg/kg. A river water contamination, which considered direct aerial deposition and soil drainage water, of 0.02 ng/L was predicted. A higher predicted water concentration of 300 ng/L was reported for surface run off water from roads (Johnson and Park, 2012).

1.6 Fate of CeO₂ NP in the environment

The potential behavior of CeO₂ NP in aquatic systems can include agglomeration, aggregation, sedimentation, dispersion and dissolution (Hardman, 2006). These processes depend on the physicochemical properties of CeO₂ NP and are as well influenced by environmental parameters such as pH, the presence of anions and cations and natural organic matter (NOM) (Handy et al., 2008).

The colloidal stability of CeO₂ NP is mostly governed by the electrostatic repulsion, which is turn depended on pH and ionic strength. The repulsion of particles depends on the surface charge which is represented by the zeta potential. CeO₂ NP with a strong negative or positive surface charge are stable in aqueous media (Berg et al., 2009). Anions and cations can suppress the surface charge resulting in agglomeration of CeO₂ NP (Buettner et al., 2010). On the other hand the sorption of phosphate to the surface of CeO₂ NP caused a decrease of zeta potential and thus stabilized CeO₂ NP in suspension (Cornelis et al., 2010). Phosphate is
known to show a strong adsorption to CeO$_2$ NP. A sorption of 0.3–0.4 mg of phosphate per gram of CeO$_2$ NP was measured by Recillas et al. (2012).

The zeta potential of CeO$_2$ NP depends strongly on the pH of the suspensions, a low pH results in positively charged CeO$_2$ NP whereas a high pH results in negatively charges CeO$_2$ NP, due to the excess of H$^+$ and OH$^-$, respectively (Hotze et al., 2010). For each system, CeO$_2$ NP have a neutral surface charge at a certain pH, which is called the isoelectric point (IEP). The IEP was determined to be often in the physiological range and causes agglomeration in ecotoxicological test and natural systems (Keller et al., 2010; Manier et al., 2013). The presence of multiple charged anions and cations can lead to the compression of the electrical double layer on the surface of CeO$_2$ NP resulting in unstable CeO$_2$ NP suspensions (Jiang et al., 2009). Thus an agglomeration of CeO$_2$ NP in natural waters seems to be unavoidable. A removal of CeO$_2$ NP from the water phase due to agglomeration followed by sedimentation was shown in mesocosm studies (Lu et al., 2010; Zhang et al., 2012).

Nanoparticles entering a natural aquatic system will interact with natural colloids, which in turn affect the behavior and fate. An agglomeration and sedimentation of CeO$_2$ NP with natural colloids is likely to occur (Klaine et al., 2008). Only a few studies have investigated the fate of CeO$_2$ NP in natural systems. A heteroaggregation of CeO$_2$ NP with natural colloids occurring in unfiltered natural water was reported to be the main mechanisms for sedimentation of CeO$_2$ NP. In filtered river water CeO$_2$ NP remained partly suspended for 12 days due to adsorption of natural organic matter (NOM) (Quik et al., 2012). The NOM fraction of natural colloids, which consists of relatively small organic compounds such as humic and fulvic acids, increases the stability of CeO$_2$ NP by a steric or electrostatic repulsion. In a model fresh water NOM stabilized CeO$_2$ NP over 12 days by decreasing the surface charge (Quik et al., 2010; van Hoecke et al., 2011). In different natural waters, ranging from groundwater to seawater the surface charge and thus the stability of CeO$_2$ NP was mainly governed by the presence of NOM and ionic strength, but was independent of pH. While with increasing NOM concentration the surface charge became more negative an increasing ionic strength neutralized the surface charge of the NOM coated CeO$_2$ NP (Keller et al., 2010). In general, the agglomeration of CeO$_2$ NP leads to larger particles, which may be transported to the sediments and probably represent the main sink of CeO$_2$ NP.
1.7 Effects of CeO$_2$ NP and cerium(III) to algae

For algae, only little data is available on the toxicity of CeO$_2$ NP and cerium(III) in literature. In general, the toxicity of CeO$_2$ NP on algae can derive from direct and indirect effects. Direct effects on organisms can be determined by internalization and surface reactivity of CeO$_2$ NP. Indirect effects can derive from release of cerium(III), production of ROS, shading of algae or from binding nutrients to the particle surface. Thus differentiation between direct and indirect effects of CeO$_2$ NP to algae is required in order to assess the cause of toxicity.

Beside one study which reports no effects of CeO$_2$ NP to algae (Velzeboer et al., 2008), different other studies report on growth inhibition of algae in a similar concentration range from 4.4 – 29.6 mg/L CeO$_2$ NP (Manier et al., 2013; Manier et al., 2011; Rodea-Palomares et al., 2011; Rogers et al., 2010; van Hoecke et al., 2009). These studies tested CeO$_2$ NP with different nominal sizes, agglomeration states as much as different dispersal protocols. It was found that with the decrease of the nominal particle diameter the toxicity to algae increased, even though CeO$_2$ NP agglomerated during exposure (van Hoecke et al., 2009). In contrast the size of agglomerates of CeO$_2$ NP seemed not to influence the effects as it was shown by testing different dispersion protocols and aged suspension (Manier et al., 2013; Manier et al., 2011). A dispersion of CeO$_2$ NP with NOM resulted in smaller particle size but with increasing NOM concentrations the toxicity decreased (van Hoecke et al., 2011). A dispersion of CeO$_2$ NP was also achieved with humic acids (HA) as reported by Manier et al., but did not affect the toxicity to P. subcapitata (Manier et al., 2011).

Some studies suggest that toxicity of CeO$_2$ NP derives from the formation of ROS which can induce oxidative damage in algae. An increase of the Ce(III)/Ce(IV) ratio was reported upon contact with algae (Rogers et al., 2010) and bacteria (Zeyons et al., 2009) and this change of redox state caused the formation of ROS. The oxidative activity of CeO$_2$ NP was shown to be involved in the production of hydroxyl radicals (Rogers et al., 2010). Different studies suggest that the observed decrease of photosynthetic yield, lipid peroxidation and a decrease membrane integrity was based on oxidative stress (Rodea-Palomares et al., 2012; Rogers et al., 2010). These studies proposed that a direct contact of CeO$_2$ NP with algae is required to induce toxicity. The direct contact can in turn induce the change of redox state on the surface of CeO$_2$ NP. The direct contact of CeO$_2$ NP with algae is promoted by the flocculation of algae and the clustering of CeO$_2$ NP on the algae cell surface as it was reported by
different studies. It is not clear whether the clustering of algae with CeO₂ NP can induce direct or indirect effects (Rodea-Palomares et al., 2011; Rodea-Palomares et al., 2012; Rogers et al., 2010; van Hoecke et al., 2009). Algae producing exopolymeric substances can protect the cell from a direct contact and thus reduce the impact of CeO₂ NP (Quigg et al., 2013). A cyanobacteria producing EPS was shown to be less sensitive to CeO₂ NP than bacteria without EPS (Zeyons et al., 2009). The membrane damage, cell disruption and the attachment of CeO₂ NP to the cell surface implies a possible uptake of CeO₂ NP in algae. The uptake of CeO₂ NP into algae cells was so far not carefully investigated. Microscopic observations and elemental mapping found no evidence for an uptake of CeO₂ NP into algae (Rodea-Palomares et al., 2011; van Hoecke et al., 2009; Zeyons et al., 2009).

Indirect effects on algae, by the release of cerium(III) from CeO₂ NP are also possible. Cerium(III) was shown to be toxic to algae and cyanobacteria in a lower concentration range as CeO₂ NP (Evseeva et al., 2010; Tai et al., 2010). In _Euglena_, cerium was mainly found to be located in the chlorophyll molecules (Ren et al., 2007). In cyanobacteria, cerium(III) increased the activity of different antioxidant enzymes and caused oxidative stress (Wang et al., 2012). However, in most studies the cerium(III) concentration in CeO₂ NP suspension was too low and thus it was excluded that effects were caused by cerium(III) (Rodea-Palomares et al., 2011; van Hoecke et al., 2009).
1.8 Scope of the thesis

The main scope of this thesis was to investigate the colloidal stability of CeO$_2$ NP in culture media for algae and to examine the interaction of CeO$_2$ NP with the green alga *Chlamydomonas reinhardtii*. The effects of CeO$_2$ NP to *C. reinhardtii* were assessed in short and long term exposure. Effects of CeO$_2$ NP mediated by dissolved cerium(III) were investigated by conducting experiments with Ce(NO$_3$)$_3$. The role of the cell wall on the effects and interaction of CeO$_2$ NP and cerium(III) was investigated by using both, the wild type and cell wall free mutant of *C. reinhardtii*.

The first objective (chapter 2) of this work was to assess the fate of CeO$_2$ NP in algae media and effects of CeO$_2$ NP to algae. The factors causing agglomeration and dispersion of CeO$_2$ NP in algae growth media were examined. Furthermore the amount of dissolved cerium(III) co-occurring in CeO$_2$ NP was assessed. The effects of agglomerated and dispersed CeO$_2$ NP on the wild type and cell wall free mutant were tested upon short term exposure. Experiments were also performed with Ce(NO$_3$)$_3$ as source for cerium(III) to account for effects which derive from dissolved fraction and not from CeO$_2$ NP *per se*. Assessed endpoints included photosynthetic yield and intracellular level of reactive oxygen species. Additionally algae cells were microscopically examined for morphological changes. This study has been submitted to Aquatic Toxicology.

The second objective (chapter 3) was to examine the cerium uptake in *C. reinhardtii* upon exposure to CeO$_2$ NP and cerium(III). The relationship of intracellular cerium concentrations to effects were examined by measuring photosynthetic yield. To gain information on the role of the cell wall, experiments were conducted with the cell wall free mutant of *C. reinhardtii*. Competition experiments were carried out to examine whether the uptake of cerium(III) occurs via calcium uptake routes.

The third objective (chapter 4) aimed to investigate the long term effects of CeO$_2$ NP on *C. reinhardtii*. To ensure exponential growth of algae over five days and to avoid indirect effects, exposure medium was changed daily. Growth, cell volume, photosynthetic yield and ATP content were examined for every subculture. Long term exposure was also performed with the cell wall free mutant to examine whether the direct contact of CeO$_2$ NP with the cell membrane induces higher toxicity.
1.9 References


List of Manufactured Nanomaterials and List of Endpoints for Phase One of the OECD Testing: Revision Programme.Series on the Safety of Manufactured Nanomaterials No. 27, Paris, France.


Chapter 2

Influence of agglomeration of cerium oxide nanoparticles and speciation of cerium(III) on short term effects to the green algae Chlamydomonas reinhardtii

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2.1 Abstract

Cerium oxide nanoparticles (CeO$_2$ NP) are increasingly used in industrial applications and may be released to the aquatic environment. The fate of CeO$_2$ NP and effects on algae are largely unknown. In this study, the short term effects of CeO$_2$ NP in two different agglomeration states on the green algae Chlamydomonas reinhardtii were examined. The role of dissolved cerium(III) on toxicity, its speciation and the dissolution of CeO$_2$ NP were considered. The role of cell wall of C. reinhardtii as a barrier and its influence on the sensitivity to CeO$_2$ NP and cerium(III) was evaluated by testing both, the wild type and the cell wall free mutant of C. reinhardtii.

Characterization showed that CeO$_2$ NP had a surface charge of ~0 mV at physiological pH and agglomerated in exposure media. Phosphate stabilized CeO$_2$ NP at pH 7.5 over 24 hours. This effect was exploited to test CeO$_2$ NP dispersed in phosphate with a mean size of 140 nm and agglomerated in absence of phosphate with a mean size of 2000 nm. The level of dissolved cerium(III) in CeO$_2$ NP suspensions was very low and between 0.1-27 nM in all tested media.

Exposure of C. reinhardtii to Ce(NO$_3$)$_3$ decreased the photosynthetic yield in a concentration dependent manner with EC$_{50}$ of 7.5 ± 0.84 μM for wild type and EC$_{50}$ of 6.3 ± 0.53 μM for the cell wall free mutant. The intracellular level of reactive oxygen species (ROS) increased upon exposure to Ce(NO$_3$)$_3$ with effective concentrations similar to those inhibiting photosynthesis. The agglomerated CeO$_2$ NP caused a slight decrease of photosynthetic yield at the highest concentrations (100 μM), while no effect was observed for dispersed CeO$_2$ NP. The low toxicity of agglomerated CeO$_2$ NP was attributed quantitatively to Ce$^{3+}$ ions co-occurring in the nanoparticle suspension whereas for dispersed CeO$_2$ NP, dissolved Ce$^{3+}$ was precipitated with phosphate and not bioavailable. Furthermore CeO$_2$ NP did not affect the intracellular ROS level. The cell wall free mutant and wild type of C. reinhardtii showed the same sensitivity to CeO$_2$ NP and Ce(NO$_3$)$_3$, indicating a minor role of the cell wall on toxicity. For both algae strains, a flocculation of cells was observed upon exposure to agglomerated CeO$_2$ NP and Ce(NO$_3$)$_3$, only algae exposed to agglomerated CeO$_2$ NP were tightly packed in exopolymeric substances.
2.2 Introduction

Cerium is a rare earth element and is used in a broad array of industrial products, such as polishing agents for glass and as an additive in stainless steel (Cook, 1990; Lu and Ives, 1995). Furthermore cerium is used in fertilizers in Chinese agriculture to improve the yield and quality of crops (Pang et al., 2002; Xu et al., 2002). In the nano particulate form, CeO₂ (CeO₂ NP) is utilized in oxygen gas sensors, solar cells and fuel cells (Corma et al., 2004; Izu et al., 2004; Murray et al., 1999). As an additive to diesel, nanoparticulate CeO₂ improves fuel combustion and decreases particulate emission in the exhaust gas (Park et al., 2008a). This widespread use in different products makes it likely that CeO₂ NP are released into the aquatic environment, where their fate and potential effects to organisms are largely unknown.

Several factors influence the fate of CeO₂ NP in aqueous systems, such as pH, ionic strength, colloids and natural organic matter (NOM). Studies investigating the fate of CeO₂ NP in different natural waters show that heteroaggregation with natural colloids and sedimentation are the main mechanisms for removal of nanoparticles from river water (Quik et al., 2012). In contrast, NOM containing fulvic and humic acids can stabilize CeO₂ NP in natural waters and in algae growth media, either by electrostatic or steric repulsion (Quik et al., 2010). Additionally pH has a significant effect on the adsorption of NOM to CeO₂ NP and therefore on the size of particle aggregates as shown by van Hoecke et al. (Van Hoecke et al., 2011). In freshwater and under conditions relevant for ecotoxicological tests CeO₂ NP tend to agglomerate, which can have effects on bioavailability and toxicity.

So far not much information is available on the effects of CeO₂ NP to algae. Inhibition of growth for the green algae *P. subcapitata* was found in different studies at a concentration range of 4.4-29.6 mg/l CeO₂ NP (Manier et al., 2013; Manier et al., 2011; Rodea-Palomares et al., 2011; Rogers et al., 2010; van Hoecke et al., 2009). In all of these studies growth inhibition was accompanied by flocculation of algae cells or by clustering of CeO₂ NP around the cell surface. Direct contact of CeO₂ NP with algae may be responsible for toxicity and was shown to cause membrane damage of *P. subcapitata* (Rodea-Palomares et al., 2011; Rogers et al., 2010). Also an increase of intracellular reactive oxygen species (ROS) was observed in algae and cyanobacteria (Rodea-Palomares et al., 2012). Light conditions used during algae assays were sufficient for CeO₂ NP to generate hydroxyl radicals and cause lipid peroxidation.
(Rogers et al., 2010). ROS was shown to be involved in toxicity of CeO$_2$ NP in mammalian cells (Auffan et al., 2009a; Park et al., 2008b) whereas other studies report on a scavenging ability of CeO$_2$ NP and a reduction of oxidative stress (Amin et al., 2011; Das et al., 2007; Xue et al., 2011). The contradictory ability of CeO$_2$ NP to both generate and scavenge ROS seems to depend on the redox state, which can change between Ce(III) and Ce(IV) (Auffan et al., 2009b) and on the oxygen vacancies on the particle surface (Korsvik et al., 2007).

In most published studies algae were exposed under conditions where CeO$_2$ NP were not stable in suspension and agglomerated to different extents during long term exposure (Rodea-Palomares et al., 2011). Even though particles agglomerate, the primary particles size was found to be relevant for toxicity. It was shown by van Hoecke et al. that growth inhibition increased with decreasing nominal particle size and that the difference in toxicity in this case was related to the surface area of CeO$_2$ NP (van Hoecke et al., 2009). In contrast to the nominal particle size, the size of agglomerates did not influence growth inhibition as much as the dispersion protocol and age of the suspension (Manier et al., 2013; Manier et al., 2011).

In most studies the level of dissolved cerium(III) in CeO$_2$ NP suspensions was low and therefore not considered to be relevant for toxicity of CeO$_2$ NP (Rodea-Palomares et al., 2011; Rogers et al., 2010; van Hoecke et al., 2009). It has been shown that dissolved cerium(III) is more toxic than CeO$_2$ NP at comparable concentrations and causes growth inhibition in different marine and freshwater microalgae (den Doore de Jong and Roman, 1965; Evseeva et al., 2010; Tai et al., 2010).

The aim of this study was to compare the effects of CeO$_2$ NP in different agglomeration states on *C. reinhardtii* under conditions where CeO$_2$ NP suspensions were stable. Furthermore the aim was to assess the role of dissolved cerium(III) on the toxicity. To this end the speciation of cerium(III) was considered and the level of dissolved cerium(III) was quantified. The same CeO$_2$ NP were tested in the agglomerated and the dispersed form, additionally short term exposures with Ce(NO$_3$)$_3$ were performed. Assessed endpoints were photosynthetic yield, intracellular ROS formation and cell morphology. In order to evaluate the role of the cell wall, experiments were performed using the wild type and a cell wall free mutant of *C. reinhardtii*. 
2.3 Materials and methods

2.3.1 Preparation of CeO₂ NP suspensions

Uncoated CeO₂ NP as a nanopowder from Nanograde (Staefa, Switzerland) with a primary particle size of 25 nm and a residual carbon of <0.03% were used. Cerium(III) nitrate hexahydrate (99.999%, trace metal basis) was purchased from Sigma Aldrich. For short term exposures, CeO₂ NP stock suspensions were prepared freshly 24 hours prior to the experiment with a concentration of 100 mg/l. Suspensions were dispersed by indirect sonication for 15 min with a water cooled cup sonicator (BB 6 with SONOPULS HD 2200, BANDELIN electronic GmbH & Co. KG, Berlin, Germany). As exposure media, MOPS buffer (10 mM, pH 7.5), MOPS buffer with 50 µM K₂HPO₄ (MOPS+PO₄) and a modified algae growth medium Talaquil, which was 1:10 diluted w/o phosphate (mod. Talaquil) were used (composition in supporting information).

CeO₂ NP were dispersed and stabilized in MOPS+PO₄ and preparation of suspensions in MOPS+PO₄ was adjusted by letting 30 mL CeO₂ NP suspension settle overnight in a 50 ml conical centrifuge tube. Dispersed nanoparticles remaining in suspension were separated from larger agglomerates in the settled fraction by removing the top 25 mL of the suspension with a pipette and were used for experiments. Concentration and size distribution of nanoparticles remaining in suspension were determined. As the percentage of CeO₂ NP remaining in suspension is dependent on several factors such as the media composition and on the initial CeO₂ NP concentration the determination of the final concentration of CeO₂ NP in suspension is required.

2.3.2 Characterization of CeO₂ NP

The CeO₂ suspensions were characterized for particle size by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments) and by nanoparticle tracking analyses (Nanosight LM10,NTA 2.0). DLS provides the Z-average size (based on intensity mean) and size distribution, measured as hydrodynamic diameter. Further information of the agglomeration state of NP was given by the polydispersity index (PDI). Surface charge was indirectly measured as average zeta potential of the particles in suspension via electrophoretic mobility using the Zetasizer. To understand the effect of algae growth media components on the aggregation behavior of CeO₂ NP, linear additive titrations were performed with all major components (NH₄Cl, CaCl₂, NaHCO₃, K₂HPO₄, MgSO₄, MOPS buffer) of the growth
medium Talaquil (composition in supporting information) in a concentration range relevant for the medium. Titrations were performed by the autotitrator (MLP2) connected to the DLS; pH of the suspension was monitored over the titration time. In addition, the influence of pH on agglomeration of CeO₂ NP was studied over the pH range from pH 2 to pH 10. The isoelectric point (IEP) was determined in NaNO₃ (1 mM) and K₂HPO₄ (50 µM). Stability of CeO₂ suspensions in MOPS buffer (10 mM), K₂HPO₄ (50 µM) and MOPS+PO₄ was tested over 24 hours in 10 mL volume at pH 7.5, where the suspensions were prepared with concentrations of 200 mg/L (1.62 mM) CeO₂ NP and were allowed to settle for 2, 4, 7 and 24 hours. Following the sedimentation for the selected time period, the upper 5 ml of the suspension were sampled of which particle size distribution and metal content were analyzed.

The dissolved fraction of CeO₂ NP was examined by centrifugal ultrafiltration (Millipore Amicon Ultra-4 3K) through a membrane with a nominal molecular weight limit of 3 kDa. Suspensions were centrifuged for 30 min at 4000 rpm (Megafuge 1.0R, Heraeus Instruments) and the filtrate was acidified. According to calculations based on thermodynamic data (Hayes et al. 2002), dissolved Ce(IV) is expected to be <10⁻¹² at neutral pH. It is therefore assumed that dissolved species released from CeO₂ NP are Ce(III).

Dissolution of CeO₂ NP was tested at two different concentrations (10 and 100 µM) and in all media used in short term exposure. Additionally, dissolution was assessed in conditioned media, this is a cell free medium which was obtained upon culturing C. reinhardtii for two hours and removing cells by centrifugation. Dissolution of CeO₂ NP was also assessed in presence of ethylenediaminetetraacetic acid (EDTA; 10 µM). Nanoparticle suspensions were kept for two hours under the same light regime as used for the algae tests. The concentration of Ce in the filtrate was related to the total Ce concentration as determined by ICP-MS.

2.3.3 Metal analysis

The total Ce concentration (isotope ^{140}\text{Ce}) in CeO₂NP suspensions and in ultrafiltered solutions was measured by ICP-MS (Element 2 High Resolution Sector Field ICP-MS, Thermo Finnigan, Bremen, Germany). CeO₂ NP suspensions were digested with HNO₃ (65%) and H₂O₂ (30%) in a high performance microwave (ultracleave, MLS, Leutkirch, Germany).
Reliability of the measurements was controlled using specific water references (refM105A20, IFA Systems).

2.3.4 Algae cultures

The wild type of the unicellular freshwater alga *C. reinhardtii* (strain CC125) and the cell wall free mutant (strain CC400) were used in this study. Both strains were obtained from the Chlamydomonas Genetics Center (Durham, USA). Algae were grown exponentially in the inorganic growth medium Talaquil (pH 7.5) which is a model fresh water medium. Growth conditions were controlled using a High Technology Infors shaker (Infors, Bottmingen, Switzerland (23°C, 90 rpm, 120 µE m⁻² s⁻¹) and were described previously by Scheidegger et al. (Scheidegger et al., 2011). Due to a higher sensitivity to mechanical stress of the cell wall free mutant, two different centrifugation protocols were applied (10 min, 1500 rpm for the mutant and 10 min, 3000 rpm for the wild type). The cell number was counted using an electronic particle counter (Z2 Coulter Counter, Beckman Coulter, Fullerton, CA, USA; Casy Model TT, Roche, Germany).

2.3.5 Exposure media and conditions

Exponentially growing algae cells, at initial cell densities of 2x10⁵ cells / mL were exposed in 10 mL to various CeO₂ NP and Ce(NO₃)₃ concentrations (0-200 µM) for two hours under continuous light condition. The effects of CeO₂ NP to *C. reinhardtii* were plotted against measured concentrations. Tested Ce(NO₃)₃ concentrations were equivalent to 0- 86.8 mg/L Ce(NO₃)₃·6H₂O. For inoculation of algae, cultures were centrifuged to remove the growth medium. The algal pellet was carefully rinsed with the exposure medium in order to avoid a carry-over of phosphate and algal exudates. In order to avoid complexation of cerium(III), short term exposure of *C. reinhardtii* was performed in a simplified medium with MOPS buffer (pH 7.5), in which algae can maintain their photosynthetic activity up to several hours (Navarro et al., 2008). The effect of CeO₂ NP and Ce(III) on photosynthesis was assessed in both media, MOPS and MOPS+PO₄. To determine the effect of CeO₂ NP and Ce(III) on intracellular ROS level, exposures were conducted in modified Talaquil media in which MOPS was maintained at 10 mM while all salts were diluted 1:10 and phosphate was removed.

Speciation calculations were conducted for cerium(III) in all exposure media with the software Visual Minteq 3.0 (KTH Royal Institute of Technology, Stockholm, Sweden.
www2.lwr.kth.se/English/OurSoftware/vminteq), which showed that Ce$^{3+}$ ions are not dissolved if PO$_4$ is present in the media, because of precipitation of CePO$_4$(s).

2.3.6 Photosynthesis

The algal photosynthetic (PS) yield of the photosystem II was measured by fluorometry using a PHYTO-PAM (Heinz Walz GmbH) equipped with an optical unit ED-101US/MP. The PS yield reflects the efficiency of the photochemical energy conversion process. It can be calculated from the light adapted minimal fluorescence (Ft) and the light adapted maximal fluorescence (Fm$'$) induced by a short saturating pulse of light.

\[
\text{Photosynthetic Yield} = \frac{(Fm' - Ft)}{Fm'}
\]

The PS yield of exposed algae was calculated as percent of control cells. Experiments were performed in triplicates and repeated at least twice for each algae strain.

2.3.7 Intracellular ROS level

For measuring intracellular ROS levels, the dye Carboxy-H$_2$DFFDA (Invitrogen, Molecular Probes) was used. H$_2$DFFDA is a chemically reduced, acetylated form of fluorescein. The molecule is nonfluorescent and is converted into green fluorescence when the acetate groups are removed by intracellular esterases and oxidation is induced by intracellular ROS. Upon oxidation, the fluorescent DFFDA can be detected at 488 nm excitation with a 520 nm emission filter (Szivák et al., 2009). Stock solutions (1 mM) of H$_2$DFFDA were stored in aliquots in DMSO at -20°C; for each experiment the dye was prepared freshly. Detection and quantification was performed by flow cytometry Partec PAS III (Partec GmbH, Muenster, Germany) and data treatment with Partec FloMax. Experiments were conducted to optimize the H$_2$DFFDA concentration and staining time, as well as a concentration of EDTA as a permeabilization agent. As the best staining condition, 50 µM of H$_2$DFFDA and 0.5 mM EDTA final concentration were chosen to stain 2x10$^5$ cells/ mL at room temperature for 15 min. Modified Talaquil was used as exposure medium, in order to achieve a low background level of stained control cells. Experiments with the wild type of C. reinhardtii were performed in duplicates.

After exposure to CeO$_2$ NP or cerium(III), subsamples (1 mL) from each flask were taken and H$_2$DFFDA with EDTA was added in each sample. Positive (unexposed stained algae) and negative controls (H$_2$O$_2$ 0.15%) were included in each experiment and treated similarly. For
data treatment the logarithmic intensities of FL3 (red fluorescence) and FL1 (green fluorescence) were used. The CeO2 NP did not exhibit a fluorescence signal in FL1 and FL3. To ensure that only algae cells are displayed in the dot plots for data treatment, only counted events showing the red fluorescence of the chlorophyll are displayed. Cell debris and nanoparticle agglomerates could thus be excluded from analysis. Therefore, in the intensity histogram of FL3 the algal auto fluorescence was gated as RN1 and in the dot plots only events were displayed which appear in the RN1 gate. Gates for data treatment were set manually: R1 for algal cells with normal fluorescence and gate R2 for increased fluorescence. The same gates were used for data treatment of all experiments. Percentage of the cells in each gate was used to calculate the relative ROS level (%) by the formula given below:

\[
\text{relative ROS level (\%)} = \frac{\text{mean DFFDA fluo. [cerium treated]}}{\text{mean DFFDA fluo. [control]}} \times 100
\]
2.4 Results

2.4.1 Stability of CeO₂ NP suspensions

In the Talaquil medium, CeO₂ NP formed large agglomerates > 1000 nm and settled out of suspension within an hour. In order to assess the influence of growth media components on the agglomeration behavior, titrations were performed with the main components of Talaquil. The surface charge of CeO₂ NP was not strongly influenced by CaCl₂ and NH₄Cl and particles remained agglomerated with mean sizes above 1000 nm (Fig. 1a and SI Fig. 1). MgSO₄ decreased the surface potential to -40 mV but the particle agglomerates remained above 1000 nm. Addition of NaHCO₃ decreased the zetapotential to -40 mV and size to 500 nm and was attributed to the change of pH from 7 to 8.5. K₂HPO₄ decreased the zetapotential to -40 mV and size to 500 nm at a concentration of 50 µM which is equivalent to the concentration in Talaquil (Fig. 1a). CeO₂ NP remained stable with increasing concentrations of K₂HPO₄, the small error bars indicate a narrow size distribution with K₂HPO₄ and the polydispersity index was < 0.5.

Size and zetapotential of CeO₂ NP were strongly dependent on pH (Fig. 1b). A positive zetapotential of +40 mV was measured for CeO₂ NP in 1 mM NaNO₃ at pH 2 and 3 the zeta potential decreased to -35 mV with increasing pH. The isoelectric point (IEP) was between pH 7-8, where strong agglomeration occurs. In a medium with 50 µM K₂HPO₄, the IEP was shifted to pH 3 and at pH 7-8 the zetapotential was 45 mV and particle agglomerate size was less than 500 nm.

Figure 1a. Size (hydrodynamic diameter) (blue) and zetapotential (red) of CeO₂ NP suspensions as function of the algae growth media components K₂HPO₄, MgSO₄·7H₂O and CaCl₂·2H₂O at pH 7.5.
Figure 1b. Size (hydrodynamic diameter) (blue) and zetapotential (red) of CeO₂ NP suspensions as function of pH in 1 mM NaNO₃ and in 0.05 mM K₂HPO₄.

The stability of CeO₂ suspensions, dispersed in different media, was examined over 24 hours at pH 7.5 (Tab. 1). In MOPS CeO₂ NP formed agglomerates of ~2000 nm and sedimentation resulted in less than 15% of CeO₂ NP remaining after two hours. In K₂HPO₄ and in MOPS+PO₄ CeO₂ NP were much more stable. After 4h 70% and after 24h more than 50% of the CeO₂ NP were still suspended. The mean size of suspended CeO₂ NP decreased over time to 152 nm.

Table 1. Size and mass of CeO₂ NP (200 mg/L) in different media after 24 hours of sedimentation at pH 7.5. Numbers indicate mean size and percentage of CeO₂ NP that remained in suspension in the top 5 mL of the total volume of 10 mL.

<table>
<thead>
<tr>
<th>time</th>
<th>MOPS</th>
<th>K₂HPO₄</th>
<th>MOPS (10 mM) + K₂HPO₄ (50 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2h</td>
<td>mean size</td>
<td>3137 nm</td>
<td>174 nm</td>
</tr>
<tr>
<td></td>
<td>% suspension</td>
<td>15 %</td>
<td>82 %</td>
</tr>
<tr>
<td>4h</td>
<td>mean size</td>
<td>2538 nm</td>
<td>166 nm</td>
</tr>
<tr>
<td></td>
<td>% suspension</td>
<td>BDL</td>
<td>77 %</td>
</tr>
<tr>
<td>7h</td>
<td>mean size</td>
<td>2015 nm</td>
<td>165 nm</td>
</tr>
<tr>
<td></td>
<td>% suspension</td>
<td>BDL*</td>
<td>77 %</td>
</tr>
<tr>
<td>24h</td>
<td>mean size</td>
<td>1954 nm</td>
<td>154 nm</td>
</tr>
<tr>
<td></td>
<td>% suspension</td>
<td>BDL*</td>
<td>63 %</td>
</tr>
</tbody>
</table>

*BDL: below detection limit

Concentration of dissolved cerium(III) was very low for all CeO₂ NP suspensions and within a range of 0.1-27 nM, i.e. 0.01-0.11% of total cerium (Tab. 2). With addition of EDTA the level of dissolved cerium(III) was higher within a range of 59-635 nM, i.e. 0.47-1.13%. Between the tested media there was no difference in the level of dissolved cerium(III) whereas the
relative level of dissolved cerium(III) was slightly higher in the lower concentrated CeO₂ NP suspensions with 10 µM compared to CeO₂ NP suspensions with 100 µM.

Table 2. Dissolution of CeO₂ NP in experimental media expressed in % of total cerium. Media were tested as conditioned* and with addition of 4 mM EDTA.

<table>
<thead>
<tr>
<th>medium</th>
<th>CeO₂ NP 10 µM</th>
<th>CeO₂ NP 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS</td>
<td>0.05 ± 0.07 %</td>
<td>0.02 ± 0.02 %</td>
</tr>
<tr>
<td>cond. MOPS</td>
<td>0.02 ± 0.02 %</td>
<td>0.01 ± 0.02 %</td>
</tr>
<tr>
<td>MOPS+EDTA</td>
<td>1.13 ± 0.00 %</td>
<td>0.53 ± 0.05 %</td>
</tr>
<tr>
<td>MOPS+PO₄</td>
<td>BDL**</td>
<td>BDL**</td>
</tr>
<tr>
<td>cond. MOPS+PO₄</td>
<td>0.01 ± 0.02 %</td>
<td>0.01 ± 0.01 %</td>
</tr>
<tr>
<td>MOPS+PO₄+EDTA</td>
<td>0.71 ± 0.01 %</td>
<td>0.47 ± 0.04 %</td>
</tr>
<tr>
<td>mod. Talaquil</td>
<td>0.11 ± 0.04 %</td>
<td>0.02 ± 0.00 %</td>
</tr>
<tr>
<td>cond. mod. Talaquil</td>
<td>0.05 ± 0.03 %</td>
<td>0.05 ± 0.01 %</td>
</tr>
<tr>
<td>mod. Talaquil + EDTA</td>
<td>1.85 ± 0.30 %</td>
<td>0.45 ± 0.04 %</td>
</tr>
</tbody>
</table>

*Conditioned medium: cell free medium which was obtained upon culturing C. reinhardtii for two hours and removing cells by centrifugation

**BDL below detection limit

2.4.2 Effect on photosynthetic yield and intracellular ROS level

Based on the experiments examining the stability of CeO₂ NP suspensions, different exposure media were used for short term toxicity experiments in which CeO₂ NP differed in agglomeration state. In MOPS buffer, CeO₂ NP formed agglomerates of ~2000 nm (CeO₂ NPagglo). In MOPS+PO₄ CeO₂ NP were dispersed (CeO₂ NPdisp) with a mean size of 140 nm and suspensions also contained smaller particles. After following the sonication protocol and a settling period of 24h, the CeO₂ NP that remained in suspension were used in exposure assessments and showed very reproducible size distributions. ICP-MS analysis showed that 50-60% of CeO₂ NP remained in this fraction. Intracellular ROS level was examined in mod. Talaquil in which CeO₂ NP agglomerated. Speciation calculations for the solubility of cerium(III) showed formation of CePO₄ (s) in the presence of phosphate, therefore exposures with cerium(III) were also carried out in phosphate free medium.

The effects of CeO₂ NP and Ce(NO₃)₃ of PS yield on C. reinhardtii wild type and cell wall free mutant are shown in Fig. 2. For CeO₂ NPagglo in MOPS buffer the PS yield showed a slight decrease to 80% of the control for concentrations above 100 µM for both algae strains.
(Fig. 2A). For CeO$_2$ NP$_{disp}$ in the MOPS+PO$_4$, no decrease of PS yield was detectable over the whole concentration range and for both algae (Fig. 2B). With increasing concentrations of Ce(NO$_3$)$_3$, PS yield of *C. reinhardtii* wild type and mutant was reduced in MOPS, with EC$_{50}$ of 7.5 ± 0.84 µM (n=2) for wild type and EC$_{50}$ of 6.3 ± 0.53 µM (n=2) for the cell wall free mutant (Fig. 2C). In MOPS+PO$_4$, the concentration response curve was shifted to higher values. A decrease of PS yield was only observed when the cerium(III) concentration exceeded the K$_2$HPO$_4$ concentration of 50 µM (Fig. 2D). Expressed as function of calculated dissolved Ce$^{3+}$, the concentration response curve approximates the one of Ce(NO$_3$)$_3$ in MOPS and toxicity is comparable (SI Fig. 2). When effects on PS yield upon CeO$_2$ NP$_{agglo}$ exposure in MOPS were analyzed as a function of dissolved cerium(III), based on data from ultrafiltration experiments (Fig. 2E and F), concentration response curve is shifted to lower values and matches with the one of Ce(NO$_3$)$_3$. 
Figure 2. Concentration-response curve of photosynthetic yield of C. reinhardtii (WT, filled circles) and cell wall free mutant (CC400, triangles) after 2 h of exposure. Photosynthetic yield values are expressed as percentage of the control. A. CeO$_2$ NP (agglomerated) in MOPS, B. CeO$_2$ NP (dispersed) in MOPS+PO$_4$, C. Ce(NO$_3$)$_3$ in MOPS, D. Ce(NO$_3$)$_3$ in MOPS+PO$_4$. CeO$_2$ NP as a function of the dissolved Ce$^{3+}$ (grey), based on the average percentage of dissolution from centrifugal ultrafiltration and total Ce(NO$_3$)$_3$ (dark) for WT (E.) and CC400 (F.).
Effects of CeO$_2$ NP$_{agglo}$ and Ce(NO$_3$)$_3$ on intracellular ROS formation were examined in modified Talaquil by staining with H$_2$DFFDA and quantification by flow cytometry (Fig. 3a). Upon exposure of the wild type *C. reinhardtii* to CeO$_2$ NP$_{agglo}$ the relative ROS level did not increase over the whole concentration range up to 200 µM, with all values within the range of the standard deviation of the control (Fig. 3b). For Ce(NO$_3$)$_3$ the effect on relative ROS level started to increase at concentrations of 2 µM and reached 494% upon exposure to 25 µM Ce(NO$_3$)$_3$. No further increase at higher concentrations was detected.
Figure 3.1. Flow cytometry dot plots showing representative example of unstained and H$_2$DFFDA stained C. reinhardtii samples and a stained positive control with H$_2$O$_2$. Dot plots are depicting FL1 (green fluorescence) versus FL3 (red fluorescence) for every single cell. Gate R1 represents algal cells with a normal fluorescence and gate R2 represents cells with an increased fluorescence due to staining. Those cells emitting higher fluorescence have an elevated level of intracellular ROS. The percentage of the algal cells in each gate was used to calculate the relative ROS level (%).

Figure 3.2. ROS production in C. reinhardtii as a function of metal concentration induced by CeO$_2$ NP (empty circles) and Ce(NO$_3$)$_3$ (filled circles). Data is expressed as relative ROS level (%).
2.4.3 *Morphology*

Confocal microscopy pictures (Fig. 4) of both algae strains showed no morphological change of cells after two hours exposure to 10 µM CeO$_2$ NP$_{agglo}$ in MOPS and CeO$_2$ NP$_{disp}$ in MOPS+PO$_4$. At 100 µM CeO$_2$ NP$_{agglo}$, cells of both strains formed flocs in which cells were tightly packed in an exopolymeric substances (EPS). For 100 µM CeO$_2$ NP$_{disp}$ algae cells remained single and very little flocculation was observed. Cells of the wild type partly had agglomerates attached to the cell surface in the flagella region. For Ce(NO$_3$)$_3$, a flocculation of both algae strains was observed in MOPS and in MOPS+PO$_4$ for concentrations of 50 and 100 µM, but not for 10 µM. Flocculation of algae during exposure to CeO$_2$ NP and Ce(NO$_3$)$_3$ was also visible by the naked eye. Dotplots of forward scatter (cell size) as a function of sideward scatter (cell complexity) showed an increase of cell complexity upon exposure to CeO$_2$ NP$_{agglo}$ for concentrations of >100 µM, whereas cell complexity remained unaffected after exposure to Ce(NO$_3$)$_3$ (SI Fig. 3).
Figure 4. Confocal microscopy images of *C. reinhardtii* wild type (left) and cell wall free mutant (right).
2.5 Discussion

2.5.1 Behavior of CeO$_2$ NP in exposure media

Agglomeration of metal oxide nanoparticles depends on surface charge which is primarily affected by pH and ionic strength. Agglomeration in Talaquil at pH 7.5 was expected considering the high ionic strength and the IEP of CeO$_2$ NP in the physiological range. Titrations with all major Talaquil components revealed that only K$_2$HPO$_4$ decreased the zeta potential and led to dispersion of CeO$_2$ NP. The stabilizing effect of K$_2$HPO$_4$ persists also in the biologically relevant pH range of 7-8 and can stabilize CeO$_2$ NP at least for 24 hours. The surface charge of CeO$_2$ NP was also decreased by MgSO$_4$, but CeO$_2$ NP remained agglomerated. Phosphate is known to quickly adsorb to CeO$_2$ NP (Recillas et al., 2012), in a similar way as it adsorbs to other metal oxides (Brown et al., 1999; Lin et al., 2011). In contrast to phosphate, it was shown that sulfate sorbs less strongly to the surface of an iron-cerium mixed oxide (Basu and Ghosh, 2013). In this study sulfate decreased the zeta potential of CeO$_2$ NP during titration, but the increasing concentration of the divalent magnesium compensated the negative surface charge by decreasing the thickness of the double layer, which depends on ionic strength of the medium (Jiang et al., 2009).

The concentration of dissolved cerium(III) measured in CeO$_2$ NP suspensions, prepared 24 hours prior the exposure, was very low. It was not influenced by different media, agglomeration state of CeO$_2$ NP or by products of algae metabolism in conditioned media. Ions released from the nanoparticle surface and measured in the aquatic phase are most likely Ce$^{3+}$, since Ce$^{4+}$ is only soluble at pH <4 and is expected to precipitate at higher pH with a solubility of $<$10$^{-12}$ M at pH 7-8 based on thermodynamic data (Hayes et al., 2002; Yu et al., 2006). The slightly higher concentration of dissolved Ce$^{3+}$ measured in presence of the strong metal binding ligand EDTA, may be due to a removal of loosely bound Ce$^{3+}$ from the nanoparticle surface. A dissolution of CeO$_2$ NP did not occur, since the level of dissolved cerium(III) in CeO$_2$ NP suspensions did not increase with time. The low level of dissolved Ce$^{3+}$ is in agreement with values reported in literature for algae and plant growth media (Rogers et al., 2010; Schwabe et al., 2013; van Hoecke et al., 2009).

2.5.2 Effect of CeO$_2$ NP and cerium(III) on photosynthetic yield and intracellular ROS level

Metal ions in suspensions of metal based nanoparticles play an important role in determining toxicity of nanoparticles. Thus, in order to evaluate short term toxicity of CeO$_2$
NP to *C. reinhardtii* we first examined the role dissolved cerium(III) had on toxicity. It was shown that Ce(NO$_3$)$_3$ inhibited photosynthesis in a concentration dependent manner in both algae strains with EC$_{50}$ values of 7.5 µM for the wild type and 6.3 µM for the cell wall free mutant. In the presence of phosphate, PS yield was not inhibited up to concentrations of 50 µM. Under these conditions cerium(III) precipitates with phosphate and PS yield was only affected when the cerium(III) concentration exceeded the phosphate concentration of 50 µM and free dissolved ionic Ce$^{3+}$ is present in the medium. Inhibition of PS yield suggested that Ce$^{3+}$ ions are bioavailable for algae and can indicate cellular internalization. However, the toxicity of Ce$^{3+}$ to PS yield is relatively low compared to other metal ions, as for instance in comparison to Ag$^+$ ions with an EC$_{50}$ of 184 nM for PS yield inhibition in the same algae (Navarro et al., 2008).

The similar sensitivity of wild type and cell wall free mutant of *C. reinhardtii* was unexpected. The cell wall acts as additional barrier for the protoplast through binding of metal ions to negatively charged sites and thereby changes uptake kinetics (Macfie et al., 1994). Thus, for cobalt, cadmium, copper and silver a higher toxicity was observed in the cell wall free mutant than the wild type of the same alga (Macfie et al., 1994; Piccapietra et al., 2012). In the case of silver, the higher sensitivity of the cell wall free algae was related to a faster and higher intracellular silver uptake (Piccapietra et al., 2012). For cerium(III) our results suggest that the cell wall has no protective function and has little influence on the uptake kinetics when considering that effect levels depend on intracellular metal concentrations.

The intracellular ROS level increased after exposure to Ce(NO$_3$)$_3$. The lowest effective concentration was similar to the effective concentration inhibiting photosynthesis. This suggests that intracellular ROS plays a role in inhibition of photosynthesis and can indicate oxidative damage. There is little data on cerium(III) toxicity and no comparable studies on effects to photosynthesis and intracellular ROS. For other algae, effect concentrations of 0.63-4.25 mg/L (1.5-30.4 µM) were reported to inhibit growth and were in the similar range as what we report here (Rodea-Palomares et al., 2011; Rogers et al., 2010).

The ability of phosphate to stabilize CeO$_2$ NP suspensions at pH 7.5 was exploited to assess toxicity of CeO$_2$ NP$_{disp}$ and compare it to the toxicity of CeO$_2$ NP$_{agglo}$. In this study, the toxicity of CeO$_2$ NP was only detected at high concentrations for the CeO$_2$ NP$_{agglo}$ but not for CeO$_2$ NP$_{disp}$. Difference in toxicity between the two agglomeration states of CeO$_2$ NP can be
explained by the different speciation of cerium(III) in the particle suspensions. As discussed above, in the presence of phosphate complexed cerium(III) is not available to algae, while it is bioavailable in absence of phosphate. Effect of CeO₂ NPₐₕ₉ₒ occurred at high concentrations that correspond to measured concentrations of cerium(III) in the suspensions which provoked similar effects on PS yield as comparable concentrations of Ce(NO₃)₃. This becomes evident when expressing the concentration response curve of CeO₂ NP as a function of the measured dissolved Ce³⁺ (Fig. 2 E+F). Other factors besides dissolved cerium(III), such as the flocculation of algae in presence of CeO₂ NP can be responsible for a slightly higher inhibition as it was measured for the cell wall free mutant. Cerium(III) was clearly shown to be more toxic than CeO₂ NP. Also other studies report a higher toxicity of cerium(III) compared to CeO₂ NP, but these studies did not consider the contribution of cerium(III) to the toxicity of CeO₂ NP (Rodea-Palomares et al., 2011; Rogers et al., 2010; van Hoecke et al., 2009). Our results indicate that CeO₂ NP do not display toxicity on algae after short term exposure independent of their agglomeration state. Similar to our findings, Velzeboer et al. found no measurable effect on photosynthesis of P. subcapitata after a short term exposure to CeO₂ NP concentrations up to 100 mg/L (580 µM) (Velzeboer et al., 2008). Other than in short term exposure, CeO₂ NP were found to inhibit photosynthesis and induce ROS at very low concentrations upon long term exposure (Rodea-Palomares et al., 2012). Contradictory results are reported with regard to the ability of CeO₂ NP to cause ROS formation or to scavenge ROS. The reactivity may depend on the ratio of Ce(III) to Ce(IV) at the particle surface (Heckert et al., 2008).

When considering the absence of toxicity of CeO₂ NP it can be assumed that CeO₂ NP are either internalized into algae cells but do not cause any harmful effect, or that CeO₂ NP are not internalized. While we initially assumed that CeO₂ NPₐₕ₉ₒ with 2000 nm particles cannot pass through the cell wall and cell membrane, uptake of smaller particles in CeO₂ NP₅ₐ₉ₒ was rather expected. The absence of toxicity in the cell wall free mutant implies that there is no uptake through the cell membrane while it is still unclear whether the cell wall also constitutes a barrier for particle uptake in algae (Behra et al., 2013).

Despite the fact that CeO₂ NP did not have effects on photosynthesis and intracellular ROS, exposure to CeO₂ NPₐₕ₉ₒ led to a flocculation of algae and to an increased cellular complexity which indicated a strong interaction between CeO₂ NPₐₕ₉ₒ and algae. In the presence of
CeO$_2$ NP agglomerates algae formed tightly packed flocs with EPS which was not observed for CeO$_2$ NP disp. Flocculation might be related to the differences in surface charge of CeO$_2$ NP agglomerates (~0 mV) and CeO$_2$ NP disp (~40 mV). Flocculation of algae cells was also observed in presence of Ce(NO$_3$)$_3$ but algae flocs were not as tight and lacked EPS. CeO$_2$ NP were previously reported to induce flocculation and a clustering of particles on the cell surface of *P. subcapitata*, whereby the interaction of CeO$_2$ NP with the cell surface also lead to an increase of cell membrane permeability (Rodea-Palomares et al., 2011; van Hoecke et al., 2009). The strong interaction of CeO$_2$ NP with the cell membrane of bacteria resulted in a reduction of Ce(IV) near the cell surface (Thill et al., 2006). Moreover, other metal based nanoparticles and carbon nanotubes were shown to induce flocculation of algae (Hartmann et al., 2010; Schwab et al., 2011). The presence of EPS might prevent the direct interaction of nanoparticles with organisms and was shown to reduce the toxicity of silver nanoparticles to a diatom (Miao et al., 2009; Zeyons et al., 2009). Thus, flocculation of algae seems to be a general response to various stresses (Rakesh et al., 2013). Whether flocculation of cells can be an indirect effect of CeO$_2$ NP and impair growth by shading or by limiting the diffusion of nutrients remains to be evaluated.

Under environmental conditions it is likely that CeO$_2$ NP agglomerate and settle out of the aqueous phase. The average cerium concentration in river water is given as 1.9 nM for freshwaters (Gaillardet, 2005) and may increase by the release of CeO$_2$ NP into the environment. Concentrations needed to cause short term effect in *C. reinhardti* are much higher in comparison to the cerium concentration in natural waters. Since CeO$_2$ NP display no toxicity to algae in our study, no harmful effects are expected to occur. Further, the small fraction of dissolved Ce$^{3+}$ released by CeO$_2$ NP is also not expected to affect algae considering the precipitation of cerium(III) with phosphate present in freshwater and the low intrinsic toxicity of cerium(III). However a dispersion of CeO$_2$ NP with NOM and the presence of strong reductants causing an increased dissolution cannot be ruled out in natural systems.
2.6 References


2.7 Supporting information

SI Figure 1. Size (hydrodynamic diameter) (blue) and zeta potential (red) of CeO$_2$ NP suspensions as function of algae growth media components NaHCO$_3$ and NH$_4$Cl at pH 7.5.

SI Figure 2. Concentration response curve of photosynthetic yield of Ce(NO$_3$)$_3$ for wild type (left) and cell wall free mutant (right) of *C. reinhardtii* in MOPS (triangles), in MOPS+PO$_4$ (circles) and in MOPS+PO$_4$ expressed as a function of calculated dissolved Ce$^{3+}$ (squares).
SI Figure 3. Dotplots of cell size (forward scatter) and as a function of cell complexity (sideward scatter) for Ce(NO₃)₃ (top line) CeO₂ NP (bottom line).
Figure 4. Size distribution of CeO$_2$ NP in Talaquil (A), in MOPS (B) measured by DLS and in MOPS+PO$_4$ (C) measured by NTA.
SI Table 1. Composition of algae growth medium Talaquil.

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<tr>
<td>MgSO₄·7H₂O</td>
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<tr>
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<td><strong>micro elements</strong></td>
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<tr>
<td>H₃BO₃</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td><strong>buffer</strong></td>
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Chapter 3

Uptake and effects of cerium(III) and cerium oxide nanoparticles to 
Chlamydomonas reinhardtii

Lena A. Röhdter, Tanja Brandt, Laura Sigg, Renata Behra

in preparation
3.1 Abstract

Cerium oxide nanoparticles (CeO₂ NP) are increasingly used in different applications. Upon their release into the aquatic environment, the exposure of aquatic organisms becomes likely. So far not much is known on the uptake of CeO₂ NP and cerium(III), which co-occurs in CeO₂ NP suspensions, into algae. In this study the uptake of CeO₂ NP and cerium(III) into the wild type and cell wall free mutant of *Chlamydomonas reinhardtii* was examined upon short term exposure. Separation of CeO₂ NP and cerium(III) not taken up or loosely bound to the cell was performed by washing algae with EDTA.

Despite a concentration and time dependent increase of cellular CeO₂ NP, the maximal cellular cerium concentration was calculated to correspond to 1.1 CeO₂ NP per cell. An internalization of CeO₂ NP with a mean size of 140 nm in *C. reinhardtii* was excluded.

For cerium(III) maximal cellular cerium concentrations of $6.04 \times 10^{-4}$ mol L<sub>cell</sub>⁻¹ and $9.0 \times 10^{-5}$ mol L<sub>cell</sub>⁻¹ in the wild type and cell wall free mutant of *C. reinhardtii* were measured. Experiments showed, that a major part of the cellular cerium in the wild type was strongly adsorbed to the cell and to not removed by EDTA. Additionally, in the wild type a competition of calcium with cerium occurred for binding sites at the cell wall but not for internalization. In both algae strains the increment of cellular cerium was shown to be linked to a strong inhibition of photosynthetic yield.
3.2 Introduction

Cerium and cerium oxide nanoparticles (CeO$_2$ NP) are increasingly used in industrial applications and consumer products (Cassee et al., 2011). Thus, a release into the aquatic environment becomes likely and consequently aquatic organisms may be exposed to cerium and CeO$_2$ NP. So far only a few studies have investigated the effects of CeO$_2$ NP and cerium(III) to algae. For CeO$_2$ NP long term effects are reported in a rather high concentration range of 4.4–29.6 mg/L CeO$_2$ NP (Manier et al., 2013; Manier et al., 2011; Rodea-Palomares et al., 2011; Rogers et al., 2010; van Hoecke et al., 2009) and in short term exposures no effects of CeO$_2$ NP to Chlamydomonas reinhardtii and Pseudokirchneriella subcapitata were found (Röhder et al., 2014; Velzeboer et al., 2008). While the mechanisms which underlie the toxicity of CeO$_2$ NP to algae are unknown, reported effects include oxidative stress due to an elevated level of reactive oxygen species (ROS) (Rodea-Palomares et al., 2012; Rogers et al., 2010) and membrane damage (Rodea-Palomares et al., 2011). Whether the toxicity of CeO$_2$ NP is linked to an increased intracellular cerium concentration has yet not been investigated. It is unclear whether nanoparticles can be internalized through the cell wall of algae.

For different metal nanoparticles the level of dissolved metal ions in suspension was shown to be relevant for toxic effects on algae (Aruoja et al., 2009; Navarro et al., 2008; Piccapietra et al., 2012). Although CeO$_2$ NP only slightly dissolve, the small fraction of dissolved cerium(III) co-occurring in CeO$_2$ NP suspensions was shown to cause harmful effects in C. reinhardtii (Röhder et al., 2014). Cerium(III) was shown to inhibit the photosynthetic yield of algae with EC$_{50}$ of 7.5 ± 0.8 µM (2h) (Röhder et al., 2014) and to cause growth inhibition in a concentration range of 0.63–4.25 mg/L (1.5–30.4 µM) (Rodea-Palomares et al., 2011; Rogers et al., 2010). The reported effects suggest an internalization of cerium(III), but so far nothing is known on the uptake of cerium(III) in algae. In general, the uptake of metal ions by algae is fast and consists of two steps. An initial rapid and passive adsorption onto the cell surface is followed by a slower internalization, involving active transport across the cell membrane (Crist et al., 1981; Monteiro et al., 2012). For cerium(III), which is a non-essential element, the active transport might occur via transporters for essential elements. The similarity of cerium to calcium, the reported ability of cerium to bind to calcium binding sites (Bentrop et al., 1997) and to inhibit the calcium transport through mitochondrial membranes (Yamada et al., 1972), indicate calcium transporters as potential pathways for cerium(III)
uptake. However, for most trivalent metals the uptake mechanisms are still speculative (Crémazy et al., 2013).

So far nothing is known on the internalization of CeO$_2$ NP and Ce(III) in algae. The cell wall of algae might represent a barrier for particle internalization. In case of silver nanoparticles with an average diameter of 29 nm no evidences for particle internalization in *C. reinhardtii* were found (Piccapietra et al., 2012). The cell wall providing adsorption sites for metal ions can also influence uptake kinetics of metal ions (Macfie and Welbourn, 2000), indirectly protecting cells from toxicity as supported by comparative studies reporting for different metals higher sensitivity of the cell wall free mutant compared to the wild type considering exposure concentrations (Macfie et al., 1994; Piccapietra et al., 2012). However, sensitivity of both strains towards Ag$^+$ ions was comparable when expressed as function of intracellular accumulated silver (Piccapietra et al., 2012). In case of Ce(III), the two algae strains displayed comparative sensitivity of photosynthesis based on exposure concentrations (Röhder et al., 2014).

In this study, the time dependence of cellular cerium accumulation in *C. reinhardtii* was examined for two concentrations of CeO$_2$ NP and Ce(III). The concentrations were selected according to the concentrations that affect photosynthesis of *C. reinhardtii* (Röhder et al., 2014). Uptake of cerium(III) was further examined in the presence of calcium in order to examine whether uptake of cerium occurs via calcium transport routes. The role of the cell wall in cerium(III) accumulation was explored by performing a comparative study with the wild type and the cell wall free mutant of *C. reinhardtii*. The two strains were compared for the sensitivity of photosynthesis towards measured cellular cerium concentrations.
### 3.3 Materials and methods

#### 3.3.1 Materials

Uncoated CeO$_2$ NP powder from Nanograde (Staefa, Switzerland) with residual carbon of <0.03% and particles a nominal size of 25 nm were used. Cerium(III)nitrate hexahydrate (99.999%, trace metal basis) was purchased from Sigma Aldrich. All chemicals were purchased in purissimum grade and stock solutions were prepared in deionized nanopure water. H$_2$O$_2$ (30%) and HNO$_3$ (65%) for acidic digestion in suprapure grade were purchased from Merck (Darmstadt, Germany). All polycarbonate and teflon containers were acid soaked in HNO$_3$ and rinsed in deionized water in order to avoid metal contaminations.

#### 3.3.2 Nanoparticle characterization

Particle size of CeO$_2$ NP suspensions was measured by nanoparticle tracking analyses (Nanosight LM10, NTA 2.0). CeO$_2$ NP size was measured in the exposure media and in presence of *C. reinhardtii*. With active swimming algae in medium, size measurement of CeO$_2$ NP were not optimal, but gave a rough estimation of the size distribution during exposure.

#### 3.3.3 Algal culture and exposure conditions

The wild type (CC125) and the cell wall free mutant (CC400) of the green algae *Chlamydomonas reinhardtii* were obtained from the Chlamydomonas Genetics Center (Durham, USA). Both strains were cultured under controlled conditions (23°C, 90 rpm, 120 µE m$^{-2}$ s$^{-1}$) in the inorganic growth medium Talaquil at pH 7.5, as previously described (Scheidegger et al., 2011). The average volume of the wild type and cell wall free mutant of *C. reinhardtii* were 150 fL and 80 fL, respectively.

Exposure of *C. reinhardtii* was done in acid washed polycarbonate Erlenmeyer. All experiments were performed with the same cell density of 2x10$^5$ cells/mL with three replicates. The cell number was counted using an electronic particle counter (Casy Model TT, Roche, Germany). Experiments with CeO$_2$ NP were done in 10 mM MOPS buffer at pH 7.5 with 50 µM K$_2$HPO$_4$ in order to keep CeO$_2$ NP particles dispersed (Röhder et al., 2014). Experiments with Ce(NO$_3$)$_3$ were done in 10 mM MOPS buffer, pH 7.5 only, to avoid complexation of cerium(III) with phosphate. CeO$_2$ NP were brought in suspension by indirect sonication (BB 6 with SONOPULS HD 2200, BANDELIN electronic GmbH & Co. KG, Berlin,
Germany) and CeO$_2$ NP suspension settled over night. For experiments only CeO$_2$ NP that remained in suspension were used (Röhder et al., 2014). Exposure time was limited to two hours in order to minimize complexation with algal exudates.

3.3.4 Wash protocols

In order to differentiate between cerium adsorbed to the cell surface of algae and internalized cerium, a wash protocol including several washings of algae with 10 mM MOPS buffer, pH 7.5 and EDTA as ligand was developed based on preliminary experiments testing various filters and number of wash steps (see supporting information). For Ce(NO$_3$)$_3$ polycarbonate membrane filters (Nuclepore Track-Etched Membranes, 0.4 µm pore size, Whatmann) showed the lowest sorption of cerium(III) compared to polypropylene membrane and cellulose nitrate filters. The wash protocol for Ce(NO$_3$)$_3$ exposed algae consisted of rinsing three times the algae on the filter with fresh medium (3x10 mL), containing 4 mM EDTA after filtration. Sorption controls were included in order to account for sorption of Ce(NO$_3$)$_3$ to the filter. Cerium(III) which is bound to algae after the wash steps is operationally defined as cellular cerium.

In order to separate CeO$_2$ NP loosely bound or in suspension from algae, filtration was done with polycarbonate filters with a pore size of 3 µm (Nuclepore Track-Etched Membranes, Whatmann). Filtration was performed by vacuum filtration using a PSF filter holder (filter holder with receiver 300-4000, Nalgene, Rochester, USA). In the filtrate no cells of *C. reinhardtii* were found, neither by cell counting nor microscopically, showing that algae did not pass through the pores. After filtration, filters were rinsed three times with fresh medium (3x10 mL), containing 4 mM EDTA. A CeO$_2$ NP control was included to account for the CeO$_2$ NP sorption to the filter. CeO$_2$ NP which were not removed by the wash steps were operationally defined as cellular cerium.

3.3.5 Uptake experiments

Exponentially growing wild type and cell wall free mutant of *C. reinhardtii* with a cell density of 2x10$^5$ cells/mL were exposed to CeO$_2$ NP (1 and 10 µM) and Ce(NO$_3$)$_3$ (1 and 10 µM). During exposure 10 mL aliquots were taken for filtration after 10, 30, 60 and 120 minutes.

In order to examine for the competition of calcium for Ce(III) uptake, experiments in presence of calcium (Ca(NO$_3$)$_2$) were performed. The concentration of Ce(NO$_3$)$_3$ was 10 µM and tested concentration of calcium were 1, 10 and 100 µM. Uptake of cerium(III) was
measured after one hour. Controls of unexposed algae and algae exposed to Ce(NO₃)₃ only, without calcium were included. The sorption of cerium(III) to filters in presence of calcium was controlled for every concentration and subtracted from the measured cellular cerium. All experiments were performed in triplicates and repeated at least twice for each algae strain.

The measured cellular cerium, which was not removed by the wash steps, was assumed to be internalized by algae and related to cell number and expressed as mol of cerium per cell volume (mol L⁻¹cell⁻¹). The uptake rates of cerium(III) for both algae strains were estimated by a linear fit of the intracellular cerium content versus time and expressed as mol L⁻¹cell⁻¹ min⁻¹. The uptake rate constant k₁ (L L⁻¹cell⁻¹ min⁻¹) was calculated assuming first order kinetics and by dividing the uptake rate with the exposure concentration (Newman and Unger, 2003). The release rate is assumed to be negligible over the time of this exposure.

\[
k_1 = \frac{\text{uptake rate (mol } L^{-1}\text{cell}^{-1} \text{min}^{-1})}{[\text{Ce}_{\text{out}}] (\text{mol } L^{-1})}
\]

3.3.6 Digestion and metal analysis

Directly after filtration the filters were transferred into Teflon tubes and digested with 4 mL of HNO₃ (65%) and 1 mL of H₂O₂ (30%) in a high performance microwave (Ultraclave, MLS, Leutkirch, Germany). To determine the exposure concentration, samples of CeO₂ NP suspensions and Ce(NO₃)₃ solutions were taken after exposure and digested. The Ce concentration (isotope ¹⁴⁰Ce) was measured by ICP-MS (Element 2 High Resolution Sector Field ICP-MS; Thermo Finnigan, Bremen, Germany). Medium, acid and filter blanks were measured in order to evaluate possible contamination. Reliability of the measurements was controlled using specific water references (refM105A20, IFA Systems). To control the wash efficiency and sorption of cerium and CeO₂ NP to the filter, control filters were included for every tested concentration and time point. Sorption controls were treated in the same way as exposed algae and finally subtracted from the measured cellular cerium.

3.3.7 Photosynthetic yield

Photosynthetic yield of both algae strains was measured fluorometrically using the PHYTO-PAM (Phyto-PAM, Heinz Walz GmbH, Effeltrich, Germany) equipped with an optical unit ED-
The photosynthetic yield was calculated from the light adapted minimal fluorescence (Ft) and the light adapted maximal fluorescence (Fm') induced by a short saturating pulse of light.

\[
\text{Photosynthetic Yield} = \frac{(Fm' - Ft)}{Fm'}
\]

The photosynthetic yield of exposed algae was calculated as percent of unexposed control cells with a photosynthetic yield of 100%. The concentration of Ce(NO₃)₃ was 10 µM and was measured at different time points from 5 min to 120 min. The photosynthetic yield in the competition experiments with Ca(NO₃)₂ was related to the control with the equivalent calcium concentration.
3.4 Results

3.4.1 Uptake of CeO₂ NP and Ce(III)

The examination of the size distribution of CeO₂ NP suspensions in the medium for uptake experiments, showed that particles had a mean size of 146 nm and a size distribution ranging from 20 to 300 nm as it can be seen in Figure 1. After two hours a mean size of 159 nm was measured and in presence of C. reinhardtii the a slightly higher mean size of 209 nm was measured (Figure 1).

![Figure 1. Size distribution of CeO₂ NP in MOPS+PO4 (pH7.5) at the beginning of uptake experiment and after two hours in presence and absence of C. reinhardtii.](image)

Upon exposure to CeO₂ NP an increase of cerium per liter cell volume in the wild type of C. reinhardtii was measured as a function of time and exposure concentration (Figure 2). Cellular cerium increased slightly upon exposure of 1 μM from 3.35 x 10⁻⁵ mol L⁻¹ to 1.32 x 10⁻⁴ mol L⁻¹ after 10 min to 4.84 x 10⁻⁴ mol L⁻¹ after 120 min. A maximal cellular cerium concentration of 4.84 x 10⁻⁴ mol L⁻¹ was measured after 120 min exposure to 10 μM CeO₂ NP.
The measured cellular cerium concentration increased with time and exposure concentration of Ce(NO$_3$)$_3$ in both strains of *C. reinhardtii* (Figure 3). For the wild type a maximal cellular cerium concentration of 6.04 x 10$^{-4}$ mol L$_{cell}^{-1}$ after two hours exposure to 10 µM Ce(NO$_3$)$_3$ was reached. Upon exposure to 1 µM Ce(NO$_3$)$_3$ a maximum cellular concentration of 2.42 x 10$^{-4}$ mol L$_{cell}^{-1}$ cerium(III) was measured (Figure 3A). The cell wall free mutant reached a maximal cellular concentration of 9.0 x 10$^{-5}$ mol L$_{cell}^{-1}$ after exposure to 10 µM Ce(NO$_3$)$_3$. Exposure to 1 µM Ce(NO$_3$)$_3$ resulted in a lower uptake with measured intracellular concentration of 5.06 x 10$^{-5}$ mol L$_{cell}^{-1}$ (Figure 3B).

Assuming that the measured cellular cerium corresponds to cerium internalized in cells uptake data were fitted linearly and uptake rate constants were compared between both algae strains. For exposure to 10 µM Ce(NO$_3$)$_3$ an uptake rate of 5 x10$^{-6}$ mol/ L$_{cell}$ min$^{-1}$ was estimated for the wild type and a lower uptake rate of 6 x10$^{-7}$ mol/ L$_{cell}$ min$^{-1}$ for the cell wall free mutant (SI Table 2). The uptake rate constant of the wild type of *C. reinhardtii* were 2 and 0.1 L L$_{cell}^{-1}$ min$^{-1}$ for the exposure concentrations of 1 and 10 µM. The cell wall free mutant showed a slower uptake of cerium(III) with rate constants of 0.3 and 0.06 L L$_{cell}^{-1}$ min$^{-1}$ for the two exposure concentrations.
Figure 3. Cellular cerium content in wild type (A) and cell wall free mutant (B) upon exposure to no (control), 1 µM and 10 µM Ce(NO₃)₃ over time.

The cellular cerium in the wild type decreased with increasing calcium concentrations (Figure 4A) from 3.19 x 10⁻⁴ mol L⁻¹ to 1.38 x 10⁻⁴ mol L⁻¹ at equimolar concentrations of calcium. At the highest calcium concentration of 100 µM cellular cerium only slightly decreased further. In contrast to the wild type, the cellular cerium content of the cell wall free mutant was not influenced by the presence of calcium in the exposure medium (Figure 4B). The cellular cerium content of the mutant changed slightly from 1.07 x 10⁻⁴ mol L⁻¹ in absence of calcium to 1.59 x 10⁻⁴ mol L⁻¹ in presence of 100 µM calcium.

Figure 4. Cellular cerium content (bars) and photosynthetic yield (circles) after 60 min for the wild type (A) and the cell wall free mutant (B) exposed to 10 µM Ce(NO₃)₃ and variable Ca(NO₃)₂ concentrations.

3.4.2 Photosynthetic yield as function of cellular cerium

The photosynthetic yield of both algae strains exposed to 10 µM Ce(NO₃)₃ decreased over time (Figure 5). After five minutes exposure the photosynthetic yield decreased to 84% for the wild type and to 82% for the cell wall free mutant. For both algae strains the strongest
inhibition of photosynthetic yield occurred within the first 30 minutes. At all measured time points the photosynthetic yield of the wild type was slightly lower than the one of the mutant. The decrease of the photosynthetic yield of both strains of *C. reinhardtii* exposed to 10 µM Ce(NO₃)₃ was expressed as function of cellular cerium (Figure 6). In both strains, the photosynthetic yield decreased with small increase of the intracellular cerium content while, in the wild type the photosynthetic yield did not further decrease with increasing cellular cerium.

![Figure 5](image1.png)

**Figure 5.** Photosynthetic yield of the wild type (circles) and cell wall free mutant (triangles) over time upon exposure to 10 µM Ce(NO₃)₃.

![Figure 6](image2.png)

**Figure 6.** Photosynthetic yield in function of cellular cerium content over time for the wild type and cell wall free mutant of *C. reinhardtii* upon exposure to 10 µM Ce(NO₃)₃.
In presence of calcium, the photosynthetic yield of the wild type changed slightly (Figure 4A). After one hour exposure to 10 µM Ce(NO₃)₃ the photosynthetic yield showed a slight increase with increasing calcium concentration from 42.2% to 53.8%. The photosynthetic yield of the cell wall free mutant did not change with increasing calcium concentrations and remained between 37% and 32% (Figure 4B).
3.5 Discussion

3.5.1 Uptake of CeO$_2$ NP in *C. reinhardtii*

Examination of uptake of CeO$_2$ NP in *C. reinhardtii* indicated that particle internalization is rather unlikely. Despite a concentration and time dependent increase of the cellular cerium, based on a particle mean size of 140 nm the measured maximal cellular cerium was calculated to correspond to a negligible number of nanoparticles per cell. After two hours exposure to 1 µM and 10 µM CeO$_2$ NP, a maximal number of 0.2 and 1.1 CeO$_2$ NP per cell were calculated, representing less than 5% of the available particles per cell (SI Table 1). These numbers most probably represent CeO$_2$ NP attached to the cell wall, which were not removed by the wash steps. In a previous study CeO$_2$ NP dispersed in MOPS+PO$_4$ were shown to attach to the flagella region of *C. reinhardtii* as it was observed microscopically for 100 µM CeO$_2$ NP (Röhder et al., 2014). For 10 µM CeO$_2$ NP a clustering in the flagella region was not microscopically visible for every cell, due to the low number of particles per cell. The absence of evidences for CeO$_2$ NP internalization is in line with a previous study, in which no effects of CeO$_2$ NP in MOPS+PO$_4$ were detectable in *C. reinhardtii* under comparable exposure conditions (Röhder et al., 2014). In fact, considering the thickness and pore size of algal cell walls (Behra et al., 2013; Navarro et al., 2008), internalization of particles in algae seems rather improbable. Also in other studies no evidence for uptake of nanoparticles into algae was found. With applying imaging methods no internalization of CeO$_2$ NP in algae and bacteria was observed (Rodea-Palomares et al., 2011; van Hoecke et al., 2009; Zeyons et al., 2009). Also for smaller fluorescent labelled gold nanoparticles of 4-5 nm no uptake into algal cells of *P. subcapitata* was detected (van Hoecke et al., 2013). In case of silver nanoparticles, the cellular silver concentrations in *C. reinhardtii*, measured by ICP-MS, were attributed to the uptake of dissolved silver co-occurring with nanoparticles in suspensions (Piccapietra et al., 2012). Altogether, the results indicate that internalization of CeO$_2$ NP does not occur in *C. reinhardtii*.

3.5.2 Uptake of cerium(III) and effects of calcium

Uptake experiments with Ce(NO$_3$)$_3$ showed cellular cerium to increase in function of time and concentration in the wild type and the cell wall free mutant of *C. reinhardtii*. Several lines of experimental evidence support that a major part of the cellular cerium measured in the wild type was strongly adsorbed to the cell wall and not internalized into algal cells. The
estimated rate constants for uptake ($k_1$) apparently indicated that the internalization of cerium into the mutant was lower than in the wild type of *C. reinhardtii*. At the same exposure concentrations the cellular cerium was lower in the mutant, which was not expected when considering the cell wall as an additional barrier for uptake. The cellular cerium concentration was operationally defined by using a metal ligand to complex and remove Ce$^{3+}$ from the cell wall. Thus the higher cellular cerium concentrations in the wild type of *C. reinhardtii* might derive from cerium which was partly bound to the cell wall and was not removed by EDTA. Similarly, Sc$^{3+}$ sorbed to cell walls of *C. reinhardtii*, was found not to be exchangeable with EDTA (Crémazy et al., 2013). The adsorption to the cell wall is the first step in metal accumulation and the removal of adsorbed metal ions from the cell wall requires the use of a strong complexing ligand (Hao et al., 1997; Hassler et al., 2004). For trivalent ions a complexing agent which is strong enough to remove adsorbed metal completely has not yet been identified (Rengel and Reid, 1997; Taylor et al., 2000).

The competition experiments with calcium showed, that in presence of a tenfold excess of calcium the cellular cerium concentration of the wild type of *C. reinhardtii* decreased only threefold, while in the cell wall free mutant the presence of calcium did not affect the cellular cerium concentration. Based on this results we conclude that calcium uptake routes were not involved in the uptake of cerium(III). An uptake of cerium(III) via calcium uptake routes was proposed due to the similarity of cerium in the trivalent state (ionic radius 1.01 Å) to bivalent calcium (ionic radius 1.00 Å) in terms of size, bonding and preferences to donor atoms (Jakupč et al., 2005). The calcium uptake into *Amaranthus* protoplasts was shown to be inhibited by the presence of cerium(III), possibly by binding to a common site at the plasma membrane (Rengel, 1994). Together the results indicate that calcium partly competes for the binding of Ce at algal cell wall and that uptake routes for Ca are unlikely involved in the internalization of cerium.

Additional evidence for the binding of Ce(III) at the cell wall of *C. reinhardtii* was provided by examining the relationship between photosynthesis and cellular cerium and the influence of Ca in that relationship. The photosynthetic yield of both algae strains exposed to 10 µM cerium(III) decreased with time. In a former study nearly similar EC$_{50}$ values of 7.5 µM and 6.3 µM of the wild type and the mutant were shown (Röhder et al., 2014). When expressing the photosynthetic yield as function of cellular cerium content it became visible, that the strong inhibition of photosynthetic yield was based on small increments of the cellular
cerium in both strains. But in the wild type only a slight decrease of photosynthetic yield was measured upon a further increase of cellular cerium, which corresponds to the cell wall bound cerium. In presence of calcium, cellular cerium concentration decreased threefold in the wild type, while the inhibitory effects on photosynthetic yield did not decrease. Whereas in the mutant the inhibitory effects on photosynthetic yield were not affected in presence of calcium. Thus, Ca$^{2+}$ rather competes for binding of Ce$^{3+}$ to cell walls than for uptake. The cellular cerium concentration of the wild type in presence of calcium was 1.16 x $10^{-4}$ mol L$_{cell}^{-1}$ and was close to the cellular cerium concentration of the mutant in absence of calcium of 1.07 x $10^{-4}$ mol L$_{cell}^{-1}$. Therefore we assume that these concentrations reflect the real cellular concentrations. Altogether, the results suggest that the measured cellular cerium concentration in the wild type includes intracellular Ce as well as Ce bound to the cell wall.

The study has demonstrated that no internalization of CeO$_2$ NP in the wild type of *C. reinhardtii* occurred. The cell wall of *C. reinhardtii* can play a dominant role on sorption of CeO$_2$ NP and cerium(III). For cerium(III) a rather slow uptake was shown, which indicated together with the high EC$_{50}$ values that no efficient transport routes for cerium(III) were available in *C. reinhardtii*. More work is needed to fully understand the interactions of trivalent ions with algae.
3.6 References


Yamada, S., Sumida, M., Tonomura, Y., 1972. Reaction Mechanism of the Ca2+-dependent ATPase of Sarcoplasmic Reticulum from Skeletal Muscle: VIII. Molecular Mechanism of the

3.7 Supporting information

Preliminary experiments with several wash steps were carried out in order to separate CeO$_2$ NP in suspension or loosely bound to the algal surface from cell bound cerium. The wash experiments showed that after three wash steps the cerium concentration per filter did not decrease any further and therefore three wash steps were applied (SI Figure 1). A successful separation of CeO$_2$ NP from algae cells was achieved with membrane filters with a pore size of 3 μm. The CeO$_2$ NP control showed that a small amount of CeO$_2$ NP remained on the filter after three wash steps.

![Graph](image)

SI Figure 1. Evaluation of the wash protocol for accumulation experiments with CeO$_2$ NP for 3, 5 and 7 wash steps on the filter with 10 ml fresh medium containing 4 mM EDTA. CeO$_2$ NP without C. reinhardtii serve as a control for sorption of CeO$_2$ NP to the filter. Cerium concentration was measured of the digested filter.
The wash experiments with Ce(NO$_3$)$_3$ showed that after one wash step with fresh media containing EDTA on the filter, the measured cerium concentration in the filter decreased by 59% after the first wash step and showed less reduction with additional wash steps. Three wash steps were performed on the filter and a sorption control without algae was included to account for the amount of cerium remaining in the filter after all wash steps (SI Figure 2).

SI Figure 2. Evaluation of the wash protocol for accumulation experiments with Ce(NO$_3$)$_3$ for 0, 1, 2, and 3 wash steps on the filter with 10 ml fresh media containing 4 mM EDTA. Ce(NO$_3$)$_3$ without C. reinhardtii serves as a control for sorption of cerium(III) to the filter. After exposure to 10 µM Ce(NO$_3$)$_3$ for 2 hours cerium concentration was measured in the digested filter.
SI Table 1. Calculated maximal number of CeO₂ NP available per cell (based on the exposure concentration and CeO₂ NP diameter) and number of CeO₂ NP sorbed on the algal cell surface after the wash procedure.

<table>
<thead>
<tr>
<th>max no. of CeO₂ NP available per cell</th>
<th>1 µM CeO₂ NP</th>
<th>10 µM CeO₂ NP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 nm</td>
<td>140 nm</td>
</tr>
<tr>
<td>10 min</td>
<td>11</td>
<td>6.4</td>
</tr>
<tr>
<td>30 min</td>
<td>12</td>
<td>0.07</td>
</tr>
<tr>
<td>60 min</td>
<td>17</td>
<td>0.1</td>
</tr>
<tr>
<td>120 min</td>
<td>37</td>
<td>0.2</td>
</tr>
</tbody>
</table>

SI Table 2. Estimated uptake rate and uptake rate constant (k₁) for cerium(III) in the wild type and cell wall free mutant of C. reinhardtii.

<table>
<thead>
<tr>
<th>exposure concentration</th>
<th>uptake rate</th>
<th>k₁</th>
<th>uptake rate</th>
<th>k₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µM Ce(NO₃)₃</td>
<td>2 x10⁻⁶ mol/L cell⁻¹ min⁻¹</td>
<td>2</td>
<td>3 x10⁻⁷ mol/L cell⁻¹ min⁻¹</td>
<td>0.3</td>
</tr>
<tr>
<td>10 µM Ce(NO₃)₃</td>
<td>5 x10⁻⁶ mol/L cell⁻¹ min⁻¹</td>
<td>0.5</td>
<td>6 x10⁻⁷ mol/L cell⁻¹ min⁻¹</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Chapter 4

Long term exposure of *Chlamydo nonas reinhardtii* and the cell wall free mutant to cerium oxide nanoparticles

Lena A. Röhder, Tanja Brandt, Laura Sigg, Renata Behra

*in preparation*
4.1 Abstract

The effects of CeO$_2$ NP on the wild type and cell wall free mutant of *Chlamydomonas reinhardtii* were assessed in a long term exposure over four subsequent cultures each lasting 24 hours. After every subculture the exposure media was renewed which ensured stable exposure conditions and the algal cell density was diluted to maintain exponential algal growth.

In every subculture growth, cell volume, photosynthetic yield and ATP content was measured at different time points for both algae strains. Additionally algae were examined microscopically. CeO$_2$ NP were characterized under exposure concentrations after 17 h and 24 h and showed agglomerates of >3 µm with a zeta potential around -10 mV. The level of dissolved cerium was low in CeO$_2$ NP suspensions with a maximal value of 4.4 nM. Conditioned media, which is a cell free medium containing algal exudates, did neither influence particle size nor dissolved Ce$^{3+}$ in CeO$_2$ NP suspensions. Controls algae of both strains showed an exponential growth in all subcultures as much as a stable photosynthetic yield and ATP content. For both strains of *C. reinhardtii* exposed to 10 µM CeO$_2$ NP, growth, cell volume, photosynthetic yield and ATP content did not differ from the control, indicating that CeO$_2$ NP did not cause any harmful effect. The direct contact of CeO$_2$ NP with the cell membrane did not induce effects in the cell wall free mutant.
4.2 Introduction

Cerium oxide nanoparticles (CeO₂ NP) are used in a variety of industrial applications and consumer products, including cosmetic products, polishing agents and oxygen sensors (Hoshino et al., 2001; Izu et al., 2004; Yabe and Sato, 2003). Predominantly CeO₂ NP are employed as diesel fuel additive to increase the fuel combustion efficiency (Park et al., 2008). Engine test have shown that CeO₂ NP emitted in the exhaust gas were in the nano scale (Jung et al., 2004). By using CeO₂ NP as a diesel fuel additive, nanoparticles may become widely dispersed throughout the environment. Predicted environmental concentrations of CeO₂ NP, in river water emanating from fuel additives, are expected to be in a low range of nanograms per liter (Johnson and Park, 2012).

So far the effects of CeO₂ NP on aquatic organisms are unclear. In literature, effects of CeO₂ NP on algae are described and range from no observed effects to inhibitory concentrations. In short term experiments no toxicity of CeO₂ NP was induced for different green algae at concentrations up to 100 mg/L (Velzeboer et al., 2008). A slight effect of agglomerated CeO₂ NP on photosynthesis of C. reinhardtii was shown to be caused by cerium(III) co-occurring in CeO₂ NP suspensions (Röhder et al., 2014). On the other hand, in long term exposure growth was inhibited by CeO₂ NP with EC₅₀ values in a rather high concentration range of 4.4-29.6 mg/L (Manier et al., 2013; Manier et al., 2011; Rodea-Palomares et al., 2011; Rogers et al., 2010; van Hoecke et al., 2009).

Exposure of algae to CeO₂ NP has been reported to be accompanied with a flocculation of algae (Rodea-Palomares et al., 2011; van Hoecke et al., 2009), as also observed during short term exposure of C. reinhardtii, which showed cells packed tightly in big flocs with an exopolymeric substance (EPS) (Röhder et al., 2014). Strong interactions of CeO₂ NP with algae can cause indirect effects due to a reduced availability of light and reduced nutrient diffusion inside of algae flocs. A reduced growth of algae can also be induced by high nanoparticle concentration in the medium which can cause a shading of algae (Schwab et al., 2011). Phosphate is known to show a strong sorption to the surface of CeO₂ NP (Recillas et al., 2012) and can result in a limitation of phosphate in the medium and impaired growth. In other studies toxicity of CeO₂ NP in algae appears to be mediated by an increase of intracellular ROS leading to oxidative stress, as much as by membrane damage upon direct contact to CeO₂ NP (Rodea-Palomares et al., 2011; Rodea-Palomares et al., 2012; Rogers et
al., 2010). It is unclear whether the reported effects are induced by CeO$_2$ NP _per se_ or by indirect effects, such as nutrient limitation, flocculation and shading of algae.

The aim of the study was to examine long term effects of CeO$_2$ NP to _C. reinhardtii_. Long term exposure was performed over five days under stable exposure conditions in which exponential growth of algae was maintained. This was ensured by an exposure over subsequent cultures, which included the change of exposure medium every day. Thereby the nutrient concentration and CeO$_2$ NP concentration was maintained stable and the amount of algal exudates released into the medium was limited. A concentration of 10 µM CeO$_2$ was chosen as a compromise for a concentration known not to induce effects in short term exposures and to avoid flocculation of algae and thus indirect effects. Long term exposure was also performed with the cell wall free mutant to examine whether the direct contact of CeO$_2$ NP with the cell membrane induces higher toxicity in this algae strain, compared to the walled one. For both algae strains growth, cell volume, photosynthetic yield and ATP content were examined for every subculture, additionally exposed algae cells were microscopically examined and compared to the unexposed control culture. Furthermore the characterization of CeO$_2$ NP, including the measurement of dissolved cerium(III), was done under exposure conditions.
4.3 Materials and methods

4.3.1 Chemicals

In this study, uncoated CeO₂ NP as a nanopowder from Nanograde (Staefa, Switzerland) with a primary particle size of 25 nm and a residual carbon of <0.03% were used. Nitric acid (HNO₃, 65%) and hydrogen peroxide (H₂O₂, 30%) were suprapure chemicals from Merck (Darmstadt, Germany).

4.3.2 Characterization of CeO₂ NP during exposure

CeO₂ NP were characterized in the algae growth medium Talaquil after 17 and 24 hours and were exposed to the same illumination, temperature and rotation as during long term exposure. Dissolved cerium(III) present in CeO₂ NP suspensions was analyzed by centrifugal ultrafiltration through a membrane with a nominal molecular weight limit of 3 kDa (Millipore, Amicon Ultra-4 3K) and by ultracentrifugation (30000 rpm, 3h, ρ_{CeO₂} 7.65 g/cm³). Ultracentrifugation was done with a total volume of 9 mL and after centrifugation 1 mL of the supernatant was analyzed. To examine the influence of algae exudates on the level of dissolved cerium(III) conditioned Talaquil, in which cells were cultured for either 17 or 24 hours and removed afterwards via centrifugation, was used. Algal exudates and released products from the algae metabolism remain in the medium. After ultrafiltration and ultracentrifugation samples were acidified with 65% HNO₃. Non-centrifuged samples for the determination of total cerium were digested in a high performance microwave (ultraclave, MLS, Leutkirch, Germany) with 4 mL of 65% HNO₃ and 1 mL of 30% H₂O₂ and metal concentration was measured by ICP-MS.

Size distribution and surface charge of CeO₂ NP suspensions were analyzed by dynamic light scattering (DLS) and electrophoretic mobility using a Zetasizer (Malvern Instruments Ltd, Malvern, UK). For measurements of particle size, the polydispersity index (PDI) was used as indicator for data quality. A PDI higher than 0.5 indicates the presence of agglomerated or sedimented particles causing measurement interference. In general, measurements with a PDI above 0.5 are considered to not meet data quality criteria and thus, not suitable for size analysis with DLS.

Cerium was quantified in every subculture by HR-ICP-MS (Element 2 High Resolution Sector Field ICP-MS, Thermo Finnigan, Bremen, Germany). Samples were taken after the start of each subculture and were treated by microwave digestion prior to ICP-MS analysis.
Reliability of the ICP-MS measurements was controlled using specific water references with a known cerium concentration (refM105A20, IFA Systems).

4.3.3 Algae cultures and growth conditions

The wild type (CC125) and the cell wall free mutant (CC400) of the unicellular green algae *C. reinhardtii* were examined. Both strains were obtained from the Chlamydomonas Genetics Center (Durham, USA). Algae were grown in Talaquil, an inorganic growth medium (Scheidegger et al., 2011), and under controlled conditions in glass Erlenmeyer flasks at continuous illumination (276 µE m⁻² s⁻¹, cool white fluorescent lamps), temperature (23°C) and rotation (90 rpm) in an incubation shaker (Multitron Standard, Infors HT, Bottmingen, Switzerland). Algae cultures were growing exponentially and started with an initial cell density of 2x10⁵ cell/ml. Due to a higher sensitivity to mechanical stress of the cell wall free mutant compared to the wild type, two different centrifugation protocols were applied (10min, 1500 rpm (400 xg) for the mutant and 10 min, 3000 rpm (1600 xg) for the wild type) (Heraeus® Megafuge 1.0R, Thermo Scientific, Waltham, USA). The cell number was counted using an electronic particle counter (Casy Model TT, Roche, Germany).

4.3.4 Exposure conditions

Both algae strains were exposed over four subsequent cultures to 10 µM CeO₂ NP. Exposure concentration was renewed in every subculture and each time CeO₂ NP suspensions were prepared freshly in Talaquil. Particles were brought in suspension by 15 min indirect sonication in a cup sonicator (BB 6 with SONOPULS HD 2200, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) (Röhder et al., 2014).

Exposure was done in Talaquil and exposure conditions were the same as for culturing algae. Long term exposure of exponentially growing algae started with an initial cell density of 2x10⁵ cells/ml. After 24 hours algae were centrifuged and an aliquot of the exponentially growing algae of subculture 1 was re-inoculated into fresh exposure medium to the initial cell density of 2x10⁵ cells/ml (subculture 2). This procedure was repeated until subculture 4. In each subculture the cell number, cell volume, photosynthetic yield and ATP content were measured at different time points. Experiments were performed in triplicates and were repeated twice. The ATP content was assessed in an independent experiment with triplicate cultures.
The photosynthetic yield was measured two hours after the transfer of algae into fresh medium to examine whether algae were stressed by centrifugation and the transfer to fresh medium. For both algae strains the photosynthetic yield was high and did not differ from the other time points, indicating that algae were not stressed by handling.

4.3.5 Assessed endpoints

Cell number and average cell volume was measured with an electronic particle counter (Casy Model TT, Roche, Germany). The average specific growth rate $\mu$ of each subculture was calculated as the slope of a linear regression of the natural logarithm of the measured cell number versus time.

Photosynthetic yield of photosystem II was measured with a pulse-amplitude modulated chlorophyll fluorometer (Phyto-PAM, Heinz Walz GmbH, Effeltrich, Germany) equipped with the Optical Unit ED-101US/MP. The photosynthetic yield can be calculated from the light adapted minimal fluorescence($F_t$) and the light adapted maximal fluorescence ($F_{m'}$) induced by a short saturating pulse of light.

$$Photosynthetic\ Yield = \frac{(F_{m'} - F_t)}{F_{m'}}$$

Intracellular ATP content was quantified by using the assay kit BacTiter Glo™ based on a bioluminescence reaction (BacTiter Glo™, PROMEGA, Madison, USA). Luminescent signal was measured in white well plates with an Infinite M200 plate reader (TECAN, Männedorf, Switzerland). With a calibration curve the luminescence signal was linearly correlated to the ATP concentration (SI Figure 1). The ATP concentration per single cell be determined by normalizing the ATP concentration to the cell number, which was counted for every sample. Controls with ATP and CeO$_2$ NP were included to account for interference of CeO$_2$ NP with the assay and luminescence reaction. At the tested concentration of 10 µM CeO$_2$ NP the ATP measurements was not affected (SI Table 1)
4.4 Results

4.4.1 Characterization of CeO₂ NP

Size and zeta potential of CeO₂ NP was measured in Talaquil under exposure conditions after 17 and 24 hours (Table 1). The size of CeO₂ NP at both time points was between 1.9 μm and >3 μm with a poly dispersity index (PDI) between 0.5 and 1, indicating a low reproducibility of measurements due to polydisperse nanoparticle suspensions. In conditioned Talaquil the mean size of agglomerated CeO₂ NP was > 3 μm for both time points. Corresponding to the strong agglomeration the PDI varied between 0.4 and 1. The zeta potential of CeO₂ NP in Talaquil changed from -11.4 mV to -9.9 mV with time. In conditioned Talaquil the zeta potential was slightly more negative and changed from -16.2 mV to -14.8 mV over time.

<table>
<thead>
<tr>
<th>time</th>
<th>Talaquil</th>
<th>conditioned Talaquil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>size (nm)</td>
<td>PDI*</td>
</tr>
<tr>
<td>17h</td>
<td>3261</td>
<td>0.51</td>
</tr>
<tr>
<td>24h</td>
<td>1943</td>
<td>0.53</td>
</tr>
</tbody>
</table>

* polydispersity index
The level of dissolved cerium(III) in Talaquil and in conditioned Talaquil was measured after 17 and 24 hours (Table 2). With ultrafiltration a level of dissolved cerium of 4.4 nM for both time points was found in Talaquil, whereas in conditioned Talaquil no dissolved cerium(III) was detectable in the filtrate. The dissolved cerium(III) measured in the supernatant after ultracentrifugation in Talaquil was 0.8 nM after 17 h and 1 nM after 24 h. In conditioned Talaquil 1.5 and 1.6 nM was measured after 17 and 24 hours.

Table 2. Dissolved Ce$^{3+}$ in CeO$_2$ NP suspensions in Talaquil and conditioned Talaquil, measured by ultrafiltration and ultracentrifugation.

<table>
<thead>
<tr>
<th>time</th>
<th>Talaquil ultrafiltration</th>
<th>Talaquil ultracentrifugation</th>
<th>conditioned Talaquil ultrafiltration</th>
<th>conditioned Talaquil ultracentrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dissolved Ce$^{3+}$ (nM)</td>
<td>dissolved Ce$^{3+}$ (%)</td>
<td>dissolved Ce$^{3+}$ (nM)</td>
<td>dissolved Ce$^{3+}$ (%)</td>
</tr>
<tr>
<td>17h</td>
<td>4.4 ± 1.3</td>
<td>0.07%</td>
<td>0.8 ± 0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>24h</td>
<td>4.4 ± 2.5</td>
<td>0.07%</td>
<td>1.0 ± 0.1</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>BDL*</td>
<td>BDL*</td>
<td>1.5 ± 0.2</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>BDL*</td>
<td>BDL*</td>
<td>1.6 ± 0.3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* below detection limit
The actual exposure concentrations in Talaquil measured by ICP-MS in every replicate and subculture were in most cases lower than the nominal concentration of 10 μM and were between 1.03 and 10.12 μM CeO₂ NP (Table 3 and 4). No increase of CeO₂ NP concentrations was measured over the four subcultures.

### Table 3. Measured exposure concentrations of CeO₂ NP for each replicate and experiment of the wild type of *C. reinhardtii*.

| subculture | experiment 1 (n=4) | | experiment 2 (n=2) | | experiment 3 (n=3) |
|------------|--------------------|-----------------|--------------------|-----------------|
|            | rep1 (µM) | rep2 (µM) | rep3 (µM) | rep4 (µM) | rep1 (µM) | rep2 (µM) | rep3 (µM) | rep1 (µM) | rep2 (µM) | rep3 (µM) |
| 1          | 3.47      | 4.56        | 1.99       | 4.83       | 5.26     | 3.52        | 6.95     | 8.30     | 7.14     |
| 2          | 3.36      | 3.01        | 2.55       | 4.50       | 5.66     | 4.20        | 6.18     | 6.15     | 7.14     |
| 3          | 2.59      | 3.52        | 1.27       | 5.27       | 5.53     | 7.24        | 7.97     | 8.71     | 10.12    |
| 4          | 2.79      | 4.74        | 2.22       | 2.83       | 6.62     | 5.99        | 5.07     | 4.85     | 9.37     |

### Table 4. Measured exposure concentrations of CeO₂ NP for each replicate and experiment of the cell wall free mutant of *C. reinhardtii*.

| subculture | experiment 1 (n=2) | | experiment 2 (n=3) | | experiment 3 (n=3) |
|------------|--------------------|-----------------|--------------------|-----------------|
|            | rep1 (µM) | rep2 (µM) | rep1 (µM) | rep2 (µM) | rep3 (µM) | rep1 (µM) | rep2 (µM) | rep3 (µM) |
| 1          | 4.10      | 4.18        | 4.11       | 5.91        | 4.75       | 5.23     | 6.69     | 6.21     |
| 2          | 3.74      | 3.52        | 2.26       | 1.03        | 1.51       | 5.19     | 6.29     | 5.54     |
| 3          | 5.13      | 3.98        | 4.97       | 2.47        | 1.76       | 5.22     | 6.49     | 8.03     |
| 4          | 3.07      | 5.04        | 2.99       | 5.52        | 4.14       | 7.12     | 7.62     | 6.74     |

#### 4.4.2 Long term exposure to CeO₂ NP

Over four subsequent cultures growth, cell volume, photosynthetic yield and ATP content were examined in every subculture for both algae strains. Control algae of both strains showed a comparable growth in each subculture. The cell number of the control cultures increased within 24 hours to an average of 7.9x10⁵ ± 5.13x10⁴ cells/mL for the wild type (Figure 1A) and 7.3x10⁵ ± 6.1x10⁴ cells/mL for the cell wall free mutant (Figure 2A). In all subcultures algae of the controls were in the exponential growth with average growth rate of 0.057 ± 0.009 h⁻¹ for the wild type and 0.054 ± 0.003 h⁻¹ for the cell wall free mutant. The cell volume of both algae strains increased slightly during every subculture and the volume of the wild type varied between 150 and 250 fl (Figure 1B). The cells of the cell wall free
mutant were smaller than the wild type with a cell volume ranging between 75 and 95 fL (Figure 2B).

For both algae strains the growth of algae exposed to 10 µM CeO2 NP did not differ from the unexposed controls and average growth rates were 0.059 ± 0.009 µ·h⁻¹ for the wild type and 0.055 ± 0.003 µ·h⁻¹ for the cell wall free mutant (Table 5 and 6).

Table 5. Growth rates of wild type of C. reinhardtii for each replicate and subculture.

<table>
<thead>
<tr>
<th>subculture</th>
<th>experiment 1 (n=4)</th>
<th>experiment 2 (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rep1 (µ)</td>
<td>rep2 (µ)</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.058</td>
<td>0.054</td>
</tr>
<tr>
<td>2</td>
<td>0.068</td>
<td>0.059</td>
</tr>
<tr>
<td>3</td>
<td>0.070</td>
<td>0.069</td>
</tr>
<tr>
<td>4</td>
<td>0.053</td>
<td>0.058</td>
</tr>
<tr>
<td>exposed CeO2 NP (10 µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.056</td>
<td>0.058</td>
</tr>
<tr>
<td>2</td>
<td>0.068</td>
<td>0.060</td>
</tr>
<tr>
<td>3</td>
<td>0.069</td>
<td>0.078</td>
</tr>
<tr>
<td>4</td>
<td>0.060</td>
<td>0.067</td>
</tr>
</tbody>
</table>

Table 6. Growth rates of cell wall free mutant of C. reinhardtii for each replicate and subculture.

<table>
<thead>
<tr>
<th>subculture</th>
<th>experiment 1 (n=2)</th>
<th>experiment 2 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rep1 (µ)</td>
<td>rep2 (µ)</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.057</td>
<td>0.048</td>
</tr>
<tr>
<td>2</td>
<td>0.050</td>
<td>0.046</td>
</tr>
<tr>
<td>3</td>
<td>0.053</td>
<td>0.053</td>
</tr>
<tr>
<td>4</td>
<td>0.055</td>
<td>0.056</td>
</tr>
<tr>
<td>exposed CeO2 NP (10 µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.049</td>
<td>0.050</td>
</tr>
<tr>
<td>2</td>
<td>0.051</td>
<td>0.058</td>
</tr>
<tr>
<td>3</td>
<td>0.054</td>
<td>0.054</td>
</tr>
<tr>
<td>4</td>
<td>0.050</td>
<td>0.060</td>
</tr>
</tbody>
</table>

The cell volume of both algae strains exposed to CeO2 NP did not differ from the control algae. At all time points and in each subculture the photosynthetic yield of control algae of both strains was high with an average of 0.7 ± 0.04 for the wild type and 0.6 ± 0.02 for the cell wall free mutant (Figure 1C and 2C). The cultures exposed to CeO2 NP did not differ from the control and exhibited the same average photosynthetic yield as the controls with 0.7 ± 0.02 for the wild type and 0.6 ± 0.02 for the cell wall free mutant. A value above 0.5 is
considered as the minimal values for unstressed algae and considered as threshold for controls.

Control and exposed algae of both strains showed a constant cellular ATP content which was similar in all subcultures and did not decline upon exposure of algae to CeO$_2$ NP (Figure 1C and 2C). The wild type had an average ATP content of 0.16 ± 0.015 fmol/cell in the controls and 0.14 ± 0.02 fmol/cell in the exposed cultures. A similar ATP content of 0.16 ± 0.014 fmol/cell for the controls and exposed cultures was measured for the cell wall free mutant.

Exposed and control cultures were examined microscopically at the end of subculture 2 and 4. The cellular morphology of both strains was not affected by exposure to CeO$_2$ NP. Only some algae cells exposed to CeO$_2$ NP of both strains were clustered in flocs and some cells had agglomerates attached to the cell surface (SI Figure 2).
Figure 1. Long term exposure of the wild type of *C. reinhardtii* to 10 µM CeO$_2$ NP over four subsequent subcultures (sub 1-4). A. cell number of control (dark) and exposed cultures (white), B. cell volume of control (dark) and exposed cultures (white) and C. Photosynthetic yield (circles) and ATP content (squares) of the control and cultures exposed to CeO$_2$ NP.
Figure 2. Long term exposure of the cell wall free mutant of *C. reinhardtii* to 10 µM CeO₂ NP over four subsequent subcultures (sub 1-4). A. cell number of control (dark) and exposed cultures (white), B. cell volume of control (dark) and exposed cultures (white) and C. Photosynthetic yield (circles) and ATP content (squares) of the control and cultures exposed to CeO₂ NP.
4.5 Discussion

4.5.1 Characterization of CeO\textsubscript{2} NP

Examination of CeO\textsubscript{2} NP under exposure conditions showed that CeO\textsubscript{2} NP agglomerated strongly in Talaquil with mean sizes between 2 and 3 µm. The mean sizes measured by DLS were not quantitative since PDI values were higher than 0.5 and can only be seen as an indication for polydisperse and agglomerated nanoparticles suspensions. In the majority of studies in literature, agglomeration of CeO\textsubscript{2} NP occurred in algae test media (Manier et al., 2011; Rodea-Palomares et al., 2011; Rogers et al., 2010), depending on the pH and ionic strength of the media. Due to the presence of anions and cations in Talaquil and a pH of 7.5 which is close to the isoelectric point of CeO\textsubscript{2} NP, a low zeta potential between -7.7 mV and -17 mV was measured, resulting in weak repulsive forces and agglomeration of the particles. In conditioned Talaquil the zeta potential of CeO\textsubscript{2} NP did not change compared to fresh Talaquil. This showed that the Talaquil composition and not the algal exudates produced within 17 and 24 hours, determined the size and zeta potential of CeO\textsubscript{2} NP.

The level of dissolved Ce\textsuperscript{3+} in CeO\textsubscript{2} NP suspensions in fresh media was very low and did not change over time, which indicated no dissolution of CeO\textsubscript{2} NP. The measured dissolved Ce\textsuperscript{3+} can derive from ions loosely bound to the surface of CeO\textsubscript{2} NP. A low level of dissolved Ce\textsuperscript{3+} in CeO\textsubscript{2} NP suspensions is reported by several studies (Rogers et al., 2010; Schwabe et al., 2013; van Hoecke et al., 2009) and was also described in a previous study (Röhder et al., 2014). In contrast to fresh Talaquil no dissolved Ce\textsuperscript{3+} was detectable by ultrafiltration in conditioned media, while by ultracentrifugation a low amount of Ce\textsuperscript{3+} in conditioned was measured. This indicated that Ce\textsuperscript{3+} complexed with algal exudates did not pass the filter during ultrafiltration. The algal exudates in conditioned media did not influence the level of dissolved cerium in CeO\textsubscript{2} NP suspensions. Also EPS of biofilm showed no effect on the level of dissolved cerium(III) after 14 days (Kroll et al., 2014).
4.5.2 Long term exposure to CeO$_2$ NP

The purpose of this study was to test whether CeO$_2$ NP have an effect on C. reinhardtii in a long term exposure. Therefore particular attention was paid to the exposure design, which aimed at stable exposure conditions and maintaining algae in the exponential growth during long term exposure. By exchanging the exposure medium in every subculture no limitation of nutrients occurred and algal exudates released into the medium were removed. Furthermore, the dilution of algae to the initial cell density in every subculture allowed to maintain a low algae density. These factors can influence the outcome of long term toxicity tests with nanoparticles and metals. Different cell densities and increasing cell numbers can change the ratio of toxicant to cell and can result in a decreased toxicity (Franklin et al., 2002). The release of algal exudates can be responsible for changes in the bioavailability and toxicity of metals (Xue et al., 1988). In this study the experimental design over subsequent cultures allowed to maintain a stable exposure concentration by renewing the CeO$_2$ NP concentration at the start of every subculture. Measured cerium concentration during long term exposure showed a high variability in various subcultures as measured by ICP-MS. In general, measured concentrations were lower than the nominal concentration of 10 µM CeO$_2$ NP, indicating losses by agglomeration, sedimentation or sorption to the test vessel as well as poor reproducibility of sampling under these conditions (Handy et al., 2012). Losses of metal nanoparticles during exposure may result in lower measured concentrations and often in a poor reproducibly of experiments as it has been reported for different ecotoxicological test systems (Hartmann et al., 2010; Rosenkranz et al., 2012; Seeger et al., 2009).

As expected, the control cultures of both algae strains showed exponential growth in all subcultures which was very reproducible in two independent experiments. The exponential growth showed that algae were not limited in nutrients. The cell volumes of both algae strains were in a normal range, as the minimal volume for cell division of the wild type is around 178 fL (Umen and Goodenough, 2001). Moreover, transfer of cells from subcultures to the other did not represent a stress as determined by measuring photosynthetic yield shortly after the transfer. The photosynthetic yield after two hours was maximal and comparable to all time points and subcultures. Similar, the ATP content of both algae strains was constant in all subcultures.
In the long term exposure to 10 µM CeO₂ NP algae did not show an effect compared to the controls neither in growth, cell volume, nor the physiological endpoints photosynthetic yield and ATP content indicating that the energy household of the cells was not influenced. Also for the algae exposed to CeO₂ NP no change for all endpoints between the subcultures was observed. CeO₂ NP did not induce any harmful effects to algae of both strains, even after five days exposure. The absence of effects in this study contrasts with reported studies where CeO₂ NP showed to inhibit algal growth by 50% in the range of 4.4-29.6 mg/L and the ATP content in the range of 2.4 - 20.3 mg/l (Manier et al., 2013; Manier et al., 2011; Rodea-Palomares et al., 2011; Rogers et al., 2010; van Hoecke et al., 2009). However, these CeO₂ NP concentrations were higher than used here (1.72 mg/L) and it is unclear whether the observed effects are caused by indirect effects. Different factors can lead to indirect effects, such as a shading of algae by a high concentration of agglomerated nanoparticles in the medium, as it was shown for carbon nanotubes (Schwab et al., 2011). The fact, that in this study all endpoints of algae exposed to 10 µM CeO₂ NP were comparable to control values indicated that under the examined conditions CeO₂ NP did not cause shading effects as it is also reported for different metal nanoparticles (Aruoja et al., 2009; Hund-Rinke and Simon, 2006). Effects of CeO₂ NP can also be caused by nutrient limitation such as a depletion of phosphate in the medium due to adsorption of phosphate to the surface of CeO₂ NP. Phosphate is known to adsorb quickly to the surface of CeO₂ NP (Recillas et al., 2012). The maximum phosphate adsorption capacity of CeO₂ NP is estimated as about 1x10⁻³ mol/g on the basis of adsorption capacities of other metal oxide surfaces (Dzombak and Morel, 1990). Using this value, about 1-2x10⁻⁶ mol/L of phosphate may be adsorbed under our experimental conditions, thus only a small fraction of about 4% of the available phosphate. Inhibitory effects of CeO₂ NP on algal growth can also be caused by a flocculation of algae exposed to CeO₂ NP or a clustering of CeO₂ NP around the cell surface. A flocculation of algae was previously observed in short term exposure to higher concentrations of CeO₂ NP (Röhder et al., 2014). The formation of algae flocs with EPS, which was avoided in long term exposure by selecting a lower concentration of CeO₂ NP, can reduce the availability of light and the nutrient diffusion to the cells. In a previous study on short term exposure of C. reinhardtii to CeO₂ NP an inhibition of photosynthesis was measured for concentrations >100 µM. The decrease photosynthetic yield was shown to be determined by the dissolved cerium(III) and not by CeO₂ NP per se. In presence of phosphate, dissolved cerium(III)
complexes to CePO$_4$ (s) and is only bioavailable to algae when the cerium(III) concentration exceeds the phosphate concentration in medium (Röhder et al., 2014). Therefore in this study the low level of dissolved cerium(III) measured in Talaquil, which contains 50 µM phosphate, is not bioavailable in long term exposure. Several studies assume that toxicity of CeO$_2$ NP is related to membrane damage of algae, induced by a direct contact of CeO$_2$ NP with algae (Rodea-Palomares et al., 2011; Rogers et al., 2010; Thill et al., 2006). However, the cell wall free mutant of _C. reinhardtii_ did not show any damaging effects upon direct contact of CeO$_2$ NP with the cell membrane. The cell wall free mutant exposed to CeO$_2$ showed a normal growth, photosynthetic yield and ATP content as the control, indicating that the direct contact of agglomerated CeO$_2$ NP with the cell membrane does not cause harmful effects.

Altogether our results show, that CeO$_2$ NP did not cause effects to both strains of _C. reinhardtii_ at the examined concentration. This concentration of 10 µM CeO$_2$ NP equates to 1.72 mg/L and is relatively low compared to other studies. Our tested concentration is in the range of reported EC$_{10}$ values 0.5-3.9 mg/L for growth in continuous exposure (Manier et al., 2013; Manier et al., 2011; van Hoecke et al., 2009) and is the concentration at which effects start to occur in algae. Comparing the tested concentration of 1.72 mg/L to predicted environmental concentrations of 0.02 ng/L (Johnson and Park, 2012) no harmful effects of CeO$_2$ NP are expected to occur in the environment. A strong agglomeration, resulting in sedimentation of CeO$_2$ NP out of the aqueous phase is reported for freshwaters (Quik et al., 2012; Zhang et al., 2012) and limits the actual time for interactions of CeO$_2$ NP with organisms in the pelagic zone. Our results imply that CeO$_2$ NP do not cause harmful effects to algae, however possible indirect effects by CeO$_2$ NP should be considered, especially at higher concentrations.
4.6 References


4.7 Supporting information

![Graph showing the relationship between ATP concentration and luminescence signal.](image)

*Si Figure 1.* Cell free ATP calibration in Talaquil. Luminescence increase, measured in relative light units (rlu), is linear correlated to the ATP concentration.

*Si Table 1.* Control experiments of ATP measurements with 10 µM CeO$_2$ NP in medium to test for interference of NP with the ATP assay. Talaquil and Talaquil with CeO$_2$ NP were spiked with a constant ATP concentration (0.1 µM) at the end of each subculture and included in each measurement.

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<tr>
<th></th>
<th>WT</th>
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<th>CC400</th>
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<tr>
<td>subculture</td>
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<td>Talaquil (µM)</td>
<td>Talaquil + CeO$_2$ NP (µl)</td>
<td>Talaquil (µM)</td>
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<tr>
<td>1</td>
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<td>0.09 ± 0</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
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<td>0.14 ± 0.01</td>
<td>0.14 ± 0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.22 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
</tbody>
</table>
SI Figur 2. Fluorescence microscope images of the wild type and cell wall free mutant of *C. reinhardtii*. Images of control cultures and exposed to 10 µM CeO₂ NP at the end of subculture 2 and 4. Arrows indicate agglomerates attached to the algal surface, scale bars represent 25 µm.
Chapter 5

Outlook
Information on the agglomeration of CeO$_2$ NP in algae growth media, on the effects and uptake of CeO$_2$ NP to *C. reinhardtii* was gathered in this thesis. Furthermore the role of dissolved cerium(III) on toxicity and its uptake in *C. reinhardtii* was evaluated. Still, further research is needed and discussed in the following paragraphs.

### 5.1 Effects of CeO$_2$ NP

In algae growth and exposure media CeO$_2$ NP showed strong agglomeration. A dispersion of CeO$_2$ NP in exposure media was achieved with phosphate. However, even in the dispersed form CeO$_2$ NP had a mean size of 140 nm. As the primary particles were strongly sintered during flame spray synthesis, a dispersion to the nominal size of 25 nm was not possible. With CeO$_2$ NP of 140 nm no harmful effects in *C. reinhardtii* were found, neither occurred an internalization of CeO$_2$ NP.

Considering the size of pores of algae cell walls, the uptake and effects of particles of <5 nm should be tested. The dispersion of CeO$_2$ NP with phosphate included the precipitation of CePO$_4$. Thus, it would be of interest to have test conditions under which CeO$_2$ NP stay dispersed and at the same time cerium(III) remains in solution. Thereby the role of cerium(III) released from CeO$_2$ NP and the effect of small CeO$_2$ NP on algae could be assessed in long term exposure.

In this study no increase of the intracellular ROS level in *C. reinhardtii* upon exposure to CeO$_2$ NP was measured, while in other studies elevated ROS and oxidative stress were detected in algae (Rodea-Palomares et al., 2012; Rogers et al., 2010). It is unclear whether this difference was based on a different reactivity of CeO$_2$ NP, which might be determined by the ratio of Ce(III)/(IV) on particle surfaces. The effects of CeO$_2$ NP with variable and known ratio of Ce(III)/Ce(IV) on algae should be assessed. The change of redox state between Ce(III) and Ce(IV) can be measured by different spectroscopic techniques (XPS, XANES, PEELS).

The ratio of Ce(III)/Ce(IV) might be also important to examine the reported ATPase activity of CeO$_2$ NP, which depends on Ce(III) sites on the particle surface (Dowding et al., 2013; Hirsch Kuchma et al., 2010). The ATPase activity can potentially lead to a decrease of ATP levels and to an interference with the extracellular phosphatase excreted by algae.

A flocculation of algae was observed upon exposure to Ce(NO$_3$)$_3$ and CeO$_2$ NP. In case of CeO$_2$ NP in MOPS+PO$_4$ an attachment of CeO$_2$ NP to the flagella of *C. reinhardtii* was microscopically observed. For CeO$_2$ NP in MOPS a strong flocculation with algae packed
tightly in an exopolymeric substance (EPS) was observed. It is still unclear what causes the flocculation of algae and the attachment of CeO₂ NP to the flagella membrane and whether it is driven by electrostatic attraction. Furthermore it remains open how the release of EPS of *C. reinhardtii* was induced and whether it poses as protection mechanism of algae to reduce the direct contact of CeO₂ NP with the cell surface.

### 5.2 Effects of cerium(III)

It was shown that cerium(III) induced toxicity in *C. reinhardtii* and was also taken up by the cells. The uptake of cerium(III) was rather slow and the question remains open whether cerium(III) uptake occurs via transport routes for essential metals. Competition experiments with different essential metals like magnesium should be performed. A competition of cerium(III) with magnesium could occur since it was shown that cerium can partly substitute magnesium in the chloroplast (Hong et al., 2002; Ren et al., 2007; Ze et al., 2009a; Ze et al., 2009b).

In this study a strong sorption of cerium(III) occurred at the cell wall of *C. reinhardtii* which could not be removed with the ligand EDTA. Ligands containing an organic phosphate group could be strong enough to desorb cerium from the cell wall and should be tested. However, it has to be considered that should not be taken up into the algal cell or lyse the cell. So far no ligands are known to fully remove the sorbed cerium(III), a quantification of the cerium(III) bound to the cell wall would be required to quantify the intracellular cerium content.

### 5.3 Effects in natural aquatic systems

The fate of CeO₂ NP in natural waters was assessed by different studies and it was shown that CeO₂ NP predominantly agglomerate in natural waters and can be stabilized by NOM (Keller et al., 2010; Quik et al., 2010; Quik et al., 2012). As the sediments are likely to be the main sink of agglomerated CeO₂ NP in aquatic environments, an exposure of benthic organisms can occur. The fate of CeO₂ NP under anaerobic conditions in the sediment, which might lead to a reduction of cerium, is not known and should be examined. Furthermore the uptake and effects of CeO₂ NP to organisms living in the sediments e.g. nematodes, snails, benthic filter feeders and microbial biofilms should be examined. As agglomerated CeO₂ NP may be deposited on biofilms, particle agglomerates can be ingested by grazers which can
lead to a transfer of CeO$_2$ NP though the food chain and to possible biomagnification in the aquatic ecosystem and thus should be evaluated.
5.4 References


Acknowledgements

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