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

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ABSTRACT

Cerium (Ce) and cerium oxide nanoparticles (CeO_2 NP) are increasingly used in different applications. Upon their release into the aquatic environment, the exposure of aquatic organisms becomes likely. In this study, the uptake of CeO_2 NP and Ce^{3+} into the wild type and cell wall free mutant of *Chlamydomonas reinhardtii* was examined upon short term exposure. Separation of CeO_2 NP and Ce^{3+} not taken up or loosely bound to the cells was performed by washing algae with EDTA.

Despite a concentration and time dependent increase of cellular Ce upon exposure to CeO_2 NP with the maximal calculated Ce concentration corresponding to 1.1 CeO_2 NP per cell, an internalization of CeO_2 NP with a mean size of 140 nm in *C. reinhardtii* was excluded. In contrast, dissolved Ce^{3+} (1 and 10 µM) was taken up both in the wild type and cell wall free mutant of *C. reinhardtii,* with a linear increase of cellular Ce within 1–2 h and maximal cellular Ce of 6.04×10^{-4} mol L_{cell}^{-1} (wild type) and 9.0×10^{-5} mol L_{cell}^{-1} (cell wall free mutant). Based on competition with Ca^{2+} for Ce^{3+} uptake, on the comparison of the wild type and the cell wall free mutant and on inhibition of photosynthetic yield, we suggest that no efficient uptake routes for Ce^{3+} are available in *C. reinhardtii* and that a fraction of the cellular Ce in the wild type strongly sorbs to the algal cell wall.

1. Introduction

Cerium (Ce) and cerium oxide nanoparticles (CeO_2 NP) are increasingly used in industrial applications and consumer products (Angel et al., 2015; Montini et al., 2016). Thus, a release into the aquatic environment becomes likely and consequently aquatic organisms may be exposed to Ce and CeO_2 NP. So far, only a few studies have investigated the effects of CeO_2 NP and Ce^{3+} to algae. For CeO_2 NP, effective concentrations upon long term exposures are reported to be in a rather high concentration range of 4.4–29.6 mg/L CeO_2 NP (Angel et al., 2015; Manier et al., 2013; Manier et al., 2011; Rodea-Palomares et al., 2011; Rogers et al., 2010; Van Hoecke et al., 2009), while 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 sorption of particles to cell walls and membrane damage (Angel et al., 2015; Rodea-Palomares et al., 2011). Although some studies report the detection of particles associated or within algal cells (Booth et al., 2015; Taylor et al., 2016), it has not yet been investigated whether the toxicity of CeO_2 NP is linked to an increased intracellular particle concentration.

For various metal nanoparticles the level of dissolved metal ions in suspension was shown to be relevant for toxic effects on algae (Aruoja et al., 2009; Müller et al., 2016; Navarro et al., 2008). Although CeO_2 NPs only slightly dissolve, the small fraction of dissolved Ce^{3+} co-occurring in CeO_2 NP suspensions was shown to cause harmful effects in *C. reinhardtii* (Röhder et al., 2014). Ce^{3+} inhibits the photosynthetic yield of algae with EC_{50} of 7.5 ± 0.8 µM (2 h) (Röhder et al., 2014) and causes 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 Ce(III), but so far information on the uptake of Ce^{3+} in algae is very scarce (El-Akl et al., 2015). In general, the uptake of metal ions by algae is fast and consists

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of two steps. An initial rapid and passive adsorption onto the cell surface is followed by a slower internalization, involving transport across the cell membrane (Crist et al., 1981; Monteiro et al., 2012). For Ce^{3+}, which is a non-essential element, the transport might occur via transporters for essential elements. The similarity of Ce to calcium (Ca), and its reported ability to replace Ca^{2+} in Ca binding proteins (Bentrop et al., 1997; Bertini et al., 2001) and to inhibit the Ca^{2+} transport through mitochondrial membranes (Yamada et al., 1972) indicate Ca^{2+} transporters as potential pathways for Ce^{3+} uptake. However, for most trivalent metals knowledge on uptake mechanisms is still speculative (Crémazy et al., 2013).

So far, little is known on the internalization of CeO_2 NP and Ce^{3+} in algae (Angel et al., 2015; El-Akl et al., 2015). The cell wall of algae might represent a barrier for particle internalization. In case of AgNP with an average diameter of 29 nm no evidence for particle internalization in *C. reinhardtii* was found (Piccapietra et al., 2012). The cell wall providing adsorption sites for metal ions can also influence uptake kinetics of metals (Macfie et al., 1994; Macfie and Welbourn, 2000; Piccapietra et al., 2012), thereby indirectly protecting cells from toxicity. This is the case if sorption is sufficiently important to reduce diffusion rate across the cell wall to the membrane transporters. Comparative studies report for several metals higher sensitivity of the cell wall free mutant compared to the wild type considering exposure concentrations (Macfie et al., 1994; Navarro et al., 2008). However, sensitivity of both strains towards Ag^{+} ions was comparable when expressed as a function of intracellular accumulated silver (Piccapietra et al., 2012). In the case of Ce^{3+}, the two algal strains displayed similar sensitivity of photosynthesis based on exposure concentrations suggesting little influence of the cell wall on the uptake of Ce^{3+} in these algae (Röhder et al., 2014).

In this study, the time dependence of cellular Ce accumulation in *C. reinhardtii* was examined for two concentrations of CeO_2 NPs and Ce^{3+}. The concentrations were selected according to the concentrations known to affect photosynthesis of *C. reinhardtii* (Röhder et al., 2014). Furthermore, the uptake of Ce^{3+} was examined in the presence of Ca^{2+} in order to examine whether Ce uptake occurs via Ca transport routes. The role of the cell wall in Ce^{3+} accumulation was explored by performing a comparative study with the wild type and the cell wall free mutant of *C. reinhardtii* in which their sensitivity of photosynthesis was related to measured cellular Ce concentrations.

2. Materials and methods

2.1. Materials

Uncoated CeO_2 NP powder from Nanograde (Staefa, Switzerland) with residual carbon of < 0.03% and a nominal particle size of 25 nm were used. Ce(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_2O_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.

2.2. 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 actively swimming algae in medium, size measurement of CeO_2 NP was not optimal, but gave a rough estimation of the size distribution during exposure.

2.3. Algal culture and exposure conditions

The wild type (CC125) and the cell wall free mutant (CC400) of the green alga *Chlamydomonas reinhardtii* were obtained from the Chlamydomonas Genetics Center (Durham, USA). The cell wall less strain CC400 is an induced mutant of the CC125 strain often used as background strain for mutations. Genetic analysis showed that CC400 is the result of a single mutation (Davies and Plaskitt, 1971) and is considered to be physiologically identical to the wild type strain (Macfie et al., 1994). Both strains were cultured under controlled conditions (23 °C, 90 rpm, 120 $\mu E\, m^{-2} s^{-1}$) 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 2×10^5 cells mL^{-1} with three replicates. The cell number was counted using an electronic particle counter (Casy Model TT, Roche, Germany).

Experiments with CeO_2 NPs were done in 10 mM MOPS buffer at pH 7.5 with 50 µM K_2HPO_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 or precipitation of Ce^{3+} with phosphate. CeO_2 NPs 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. Only CeO_2 NPs remaining in suspension were used for exposures (Röhder et al., 2014). Exposure time was limited to two hours in order to minimize presence of algal exudates binding Ce^{3+}.

2.4. Wash protocols

In order to differentiate between Ce adsorbed to the cell surface of algae and internalized Ce, 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 (Supporting Information). For uptake experiments with $Ce(NO_3)_3$ polycarbonate membrane filters (Nuclepore Track-Etched Membranes, 0.4 µm pore size, Whatmann) were used as these showed the lowest sorption of Ce^{3+} compared to polypropylene membrane and cellulose nitrate filters.

The wash protocol for $Ce(NO_3)_3$-exposed algae consisted of rinsing the algae three times on the filter with fresh medium (3 × 10 mL), containing 4 mM EDTA after filtration. Sorption controls were included in order to account for sorption of Ce(III) to the filter and accounted for 0.7-11.3%. Ce(III) associated with algae after the wash steps was operationally defined as cellular Ce.

In order to separate CeO_2 NPs loosely bound or in suspension from algae, filtration was done with polycarbonate filters with a pore size of 3 µm (Nucleopore 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 (3 × 10 mL), containing 4 mM EDTA. A CeO_2 NP control was included to account for the CeO_2 NP sorption to the filter. Ce retained on filters with algae after the wash steps was operationally defined as cellular Ce.

2.5. Uptake experiments

Exponentially growing wild type and cell wall free mutant of *C. reinhardtii* with a cell density of 2×10^5 cells mL^{-1} were exposed to CeO_2 NPs (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 min.

In order to examine the competition of Ca^{2+} for Ce^{3+} uptake,