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Bacterial metabolites and particle size determine cerium oxide nanomaterial biotransformation

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Soil is a major receptor of manufactured nanomaterials (NMs) following unintentional releases or intentional uses. Ceria NMs have been shown to undergo biotransformation in plant and soil organisms with a partial Ce(IV) reduction into Ce(III) but the influence of environmentally widespread soil bacteria is poorly understood. We used high-energy resolution fluorescence detected X-ray absorption spectroscopy (HERFD-XAS) with an unprecedented detection limit to assess Ce speciation in a model soil bacterium (*Pseudomonas brassicacearum*) exposed to CeO$_2$ NMs of different sizes and shapes. The findings revealed that the CeO$_2$ NMs size drives the biotransformation process. No biotransformation was observed for the 31 nm CeO$_2$ NMs, contrary to 7 nm and 4 nm CeO$_2$ NMs, with a Ce reduction of 64 ± 14% and 70 ± 15%, respectively. This major reduction appeared quickly, from the early exponential bacterial growth phase. Environmentally relevant organic acid metabolites secreted by *Pseudomonas*, especially in the rhizosphere, were investigated. The 2-keto-gluconic and citric acid metabolites alone were able to induce a significant reduction in 4 nm CeO$_2$ NMs. The high biotransformation measured for < 7 nm NMs would affect the fate of Ce in the soil and biota.
**Synopsis**

Soil bacteria are widespread in the environment but little is known on their influence on the fate of nanomaterials. The NM size, the bacterial interaction and bacterial metabolites drive NM bioreduction.

**Introduction**

The biological transformation of nanomaterials (NMs) is a key to understanding the impact and fate of NMs in the environment.\(^1\), \(^2\) Soil is a major environmental collector for NMs, along with sediment and landfill.\(^3\), \(^4\) Soil is also a complex ecological system hosting a diverse range of living organisms and millions of microorganisms that can induce environmental NM transformation at the scale of the organisms, tissues, cells and biomolecules. Plants and microorganisms secrete secondary metabolites, i.e. specialized molecules, that are essential for their biological activities while modulating their interactions with the environment and neighboring organisms. Despite soil biodiversity, NM biotransformation in terrestrial ecosystems has primarily focused on plant-nanomaterial interfaces and several studies have assessed the fate of CeO\(_2\) NMs in the vicinity of plant roots.\(^5\)-\(^7\) Due to the electronic structure of CeO\(_2\) NMs, the Ce(IV)/Ce(III) ratio at the NM surface is variable, while the core atoms are mainly in a Ce(IV) state.\(^8\) Partial Ce reduction of CeO\(_2\) NMs was measured in several plant species such as tomato\(^9\), wheat\(^5\) and cucumber\(^10\). Owing to this versatile redox reactivity, CeO\(_2\) NM is a good candidate to assess the NM biotransformation in soil.

The root surface\(^6\), \(^9\), \(^11\), \(^12\) and root-soil interface\(^7\) are the sites of interaction between plants and NMs. Root metabolites excreted at these interfaces induce a reduction of CeO\(_2\) NMs in plant systems.\(^6\), \(^13\) Plenty of metabolites are candidates (saccharides, amino acids, organic acid, phenols, etc.), yet only citric acid and ascorbic acid were shown to reduce CeO\(_2\) NMs, through incubation experiments simulating root exudates.\(^11\) Soil and root associated bacteria also release a large set of secondary metabolites in their environment for their nutrition (dissolution and uptake of iron, phosphate, etc.), and for their communication with other microorganisms and plants. For instance, biodissolution of Au NMs has been induced by the associated microbiome of a freshwater macrophyte, despite the high chemical stability of Au NMs.\(^14\) The main driver of Au biodissolution is its oxidation and complexation with a secondary metabolite, i.e. cyanide, emitted by microorganisms.\(^14\) Regarding CeO\(_2\) NMs, their transformation by bacteria has been little addressed in the literature and the mechanism is poorly documented. Up to 50% of reduced Ce occur in *Escherichia coli* and *Synechocystis* sp. exposed to 7 nm CeO\(_2\) NMs.\(^15\), \(^16\) However, the soil bacterium *Bacillus subtilis* has proven inefficient in reducing Ce in rod-ceria 8.5 nm x.168 nm NMs, without the addition of exogenous ascorbic acid.\(^17\) The bacterial cell surface is the nano-bio interface that promotes the reduction of CeO\(_2\) NMs.\(^16\)
however the mechanisms and the potential bacterial metabolites allowing this reduction are still unknown.

What are the differences in terms of cell interface and secondary metabolism between bacteria that do (E. coli and Synechocystis sp., gram-negative bacteria) or do not reduce (B. subtilis, gram-positive bacteria) CeO$_2$ NMs? Apart from the structural differences in their outer envelope, Gram-negative and Gram-positive bacteria differ in the way they metabolize glucose, i.e. a carbon source for bacteria. Both take up glucose and metabolize it by phosphorylation to generate energy-rich molecules (e.g. ATP). However, Gram-negative bacteria can shut down this pathway and enable rapid conversion of glucose to gluconic acid, and then to 2-keto-gluconic acid instead. Gluconic acid and 2-keto-gluconic acid can both be secreted at the outer bacterial membrane surface, and later internalized to feed the cell metabolism. Gluconic acid is the preferred substrate, and is metabolized to pyruvic acid and then citric acid through the Krebs cycle. 2-keto-gluconic acid is a reserve substrate for Pseudomonas sp. and known to accumulate in the rhizosphere. This ability to rapidly convert and secrete glucose from other species, and to secrete 2-keto-gluconic acid is a key feature involved in the ecological fitness of Gram-negative bacteria, such as Pseudomonas sp., a diverse, ubiquist and ecologically significant soil bacteria. Citric acid and 2-keto-gluconic acid are important metabolites secreted by Pseudomonas sp. to solubilize phosphate and chelate iron. As they are released at the cell membrane interface, these two organic acids stand out as two potential metabolite candidates for CeO$_2$ NM biotransformation.

Key physicochemical properties of NMs, such as their shape, size and surface charge, drive their fate and their biological impact in the environment. Among octahedral, cubic, rod, and irregularly shaped CeO$_2$-NMs, rod-like particles are transformed to a greater degree than other CeO$_2$-NMs in solutions simulating plant exudates in hydroponic cucumber plants. According to the particle shape and surface chemistry, CeO$_2$ NMs exhibits both pro- and antioxidant activities, with the former being toxic to E. coli. The surface charge of CeO$_2$ NMs has been shown to affect Ce(IV) biotransformation with a partial reduction into Ce(III) in activated sludge bioreactors, in the nematode Caenorhabditis elegans, and in wheat and tomato. The size and aggregation of CeO$_2$ NMs drive the microbial activity and bacterial community structure in the canola rhizosphere. Therefore, the biotransformation of CeO$_2$ NMs depends on the NM properties but also on the tested organisms and environmental conditions. No marked relation between the size of CeO$_2$ NMs and the extent of Ce reduction in the organisms has been noted to date.

While predicted concentration of CeO$_2$ NMs in soils ranges from ng.kg$^{-1}$ to mg.kg$^{-1}$, most studies addressing Ce biotransformation used concentrations close to or higher than 100 mg.kg$^{-1}$. One of the reasons for this is the technical difficulty of detecting, quantifying and characterizing CeO$_2$ NMs at lower concentrations in complex matrices. Recent advances in X-ray absorption spectroscopy (XAS) such as high-energy resolution fluorescence detected XAS (HERFD-XAS) have enabled measurement of XANES spectra with higher energy resolution, i.e. with an apparent core hole lifetime broadening around 0.70eV, smaller than the probed electronic level bandwidth (around 3.2eV) and a lower
detection limit (as low as 60 mg Ce/kg). The better defined edge and pre-edge features in the HERFD-XAS spectra enable clear detection of the presence of Ce(III) in the structure of CeO₂-NMs. It is essential to determine the oxidation state of CeO₂ NMs so as to be able to identify the potential biotransformation of CeO₂ NMs, a process which influences their bioavailability and potential ecotoxicity.

This study aimed at identifying the biotransformation mechanisms of different sizes and shapes of CeO₂ NMs by P. brassicacearum NFM421, a phytobenefic soil bacteria isolated from Arabidopsis thaliana and Brassica napus plants. We investigated the fate of CeO₂ NMs in contact with P. brassicacearum cells in planktonic conditions, and in cell-free incubations with specific metabolites of citric acid and 2-keto-gluconic acid. Ascorbic acid was also tested as a positive control to assess Ce reduction. The bacterial metabolism and cell growth cycle are interconnected: different sets of metabolites are produced throughout the three growth phases in planktonic conditions. We therefore explored CeO₂ NM reduction by P. brassicacearum at different steps of the growth cycle (i.e. the latency phase, where cells adapt to their environment, the exponential phase, where cells divide, and the stationary phase, where cells adapt to the nutrient reduction conditions), and we tested the secretome, i.e. all metabolites excreted by the bacteria in the late exponential phase. We used HERFD-XAS and were thus able to assess changes in Ce speciation and identify the drivers of CeO₂ NMs biotransformation.

Materials and Methods

CeO₂ nanomaterials

Three different sizes of commercially available types of bare CeO₂-NMs were used in this study, all of which are made of cerianite crystallites. Transmission electron microscopy indicated average sizes of 4 nm (CeO₂-4 nm) diameter with a spherical shape (Rhodia Chemicals), 7 nm (CeO₂-7 nm) with an ellipsoidal shape (Rhodia Chemicals), and 31 nm (CeO₂-31 nm) diameter with a spherical shape (Nanograin® Umicore). These NMs have been previously described and their characteristics are summarized in the Supporting Information (Table S1). The 7 nm NMs were an ellipsoid of revolution with an average radius of the semi-axes of the ellipsoid of R = 3.5 and 0.91 nm. The specific surface areas were estimated from their size and shape: 231 m²·g⁻¹ for CeO₂-4 nm, 290 m²·g⁻¹ for CeO₂-7 nm and 30 m²·g⁻¹ for CeO₂-31 nm. The surface atom percentages were calculated using the formula given in Deshpande et al. using different lattice parameters (0.557 nm 0.553 and 0.547 nm for 4, 7 and 31 nm CeO₂ NMs). We hypothesized that the outer shell of atoms has a finite thickness of half the lattice constant. The percentages of surface atoms were approximately 36%, 41% and 5.2% for the 4 nm, 7 nm and 32 nm forms, respectively).

Bacteria and incubation conditions.

The Pseudomonas brassicacearum strain NFM421, a phytobenefic soil-bacteria, was
selected for this study. The *Pseudomonas* genus has a robust metabolism for different carbon sources, and a three-pronged metabolic system to metabolize glucose, a bacterial carbon source that can be released in the rhizosphere from plant roots. The *P. brassicacearum* genome is sequenced, thereby allowing metabolic pathway prediction. Bacterial growth involves three different main phases: i) a lag phase, i.e. a nonreplication period during which the bacterial cells adapt; ii) an exponential phase, during which cell division occurs at a constant rate, with a generation time close to 55 min for *P. brassicacearum*; and iii) the stationary phase, where the growth rate levels off at zero, while the bacteria stay metabolically active and compensate for those that die.

*P. brassicacearum* NFM421 from frozen stock (−80 °C) was grown at 30°C in 10-fold diluted tryptic soy broth (TSB 1/10) solidified with 15 g L⁻¹ agar (Sigma) under dark conditions. After 48 h, a single colony was resuspended in TSB 1/10, incubated overnight at 30°C and stirred at 150 rpm in an orbital shaker, until a concentration of 1 × 10⁹ cells per mL in stationary phase was reached, and this was used as a pre-culture.

Aliquots of the pre-culture were transferred to a new TSB 1/10 medium in order to reach an optical density at 600 nm (OD₆₀₀) of 0.05 (~ 2.10⁶ CFU/mL Figure SI 2). Bacteria and TSB/10 medium were transferred into 50 mL tubes or 1 L erlenm, stirred at 150 rpm, incubated until the early exponential growth phase was reached at OD₆₀₀ = 0.1 (~4.10⁷ CFU/mL), after approximately 60 min. Several exposures of the *P. brassicacearum* soil bacteria to different sized and shaped CeO₂ NMs were then performed:

1) Exposure to the three sizes of CeO₂ NMs: 4 nm, 7 nm and 31 nm at 5 mg CeO₂/L, from the early- to the late-exponential phase (t₃: OD₆₀₀ 0.7 (2.8 10⁸ CFU/mL)). (See SI Figure SI 3).

2) Exposure to several CeO₂-4 nm concentrations: 0.01-0.1-1.5-10 mg CeO₂/L, from the early- to the late-exponential phase (t₃). Note that the tested concentration did not affect the bacterial growth.

3) Exposure to CeO₂-7 nm at 5 mg CeO₂/L with bacterial sampling at different times during the exponential phase: t₁) OD₆₀₀ 0.15, after 45 min of contact, which was less than the generation time (cell division) for *P. brassicacearum*, t₂) OD₆₀₀ 0.3, 90 min mid-exponential phase and t₃) OD₆₀₀ 0.7, 165 min, late-exponential phase.

For each size of CeO₂ NMs, a stock solution at 0.3 g Ce/L was prepared prior to bacterial culture dosing. The bacterial culture was incubated at 30°C and stirred at 150 rpm. After the bacterial growth phase, the suspensions were centrifuged at 1500 g at 4°C to separate the bacteria and supernatant. The supernatants were discarded, and the bacterial pellets were either immediately frozen in 5 mm diameter pellets in liquid N₂ and stored at -80°C for XAS measurements, or freeze-dried for ICP-MS analysis.

The effect of the culture medium, single metabolites and secretome (i.e. all metabolites excreted by the bacteria exposed or not to CeO₂ NMs) on the Ce reduction were tested. For these analyses, only CeO₂-4 nm was tested due to time constraints for the Synchrotron analyses and because the extent of bioreduction after bacterial exposure was similar for
4 nm and 7 nm (Figure 1). The culture medium (TSB 1/10) and several commercialized metabolites (2 keto-gluconic acid, ascorbic acid, citric acid from Sigma Aldrich) were incubated with CeO$_2$-4 nm. The culture medium (TSB 1/10) and citric acid were also incubated with CeO$_2$-7 nm. Metabolite solutions were prepared at 50 mM concentration, and CeO$_2$ NMs at 300 mg Ce/L (2 mM) concentration. The metabolites were in excess compared to CeO$_2$ NMs. Solutions were adjusted to pH=7.2. The suspensions were incubated at 25°C under agitation for 24 h. After the incubation, droplets of the suspensions were frozen in liquid N$_2$ and kept at -80°C before XAS analyses.

To test the influence on the Ce reduction of the secretome, the bacterial culture supernatant was incubated with CeO$_2$ NMs. We hypothesized that the bacteria could respond to stress when exposed to NMs responsible for specialized metabolite excretion. We compared two different secretomes: the supernatant of _P. brassicacearum_ not exposed to NMs, after a bacterial growth to an OD$_{600}$ of 0.7 (same conditions as explained above), and the supernatant of _P. brassicaceae_ exposed to 5 mg.L$^{-1}$ CeO$_2$-4 nm. After the bacterial growth, the suspensions were centrifuged to separate the bacteria and supernatant. The 4-nm CeO$_2$ NMs were added to these supernatants at 300 mg Ce/L concentration and incubated under agitation for 24h. After the incubation, droplets of the suspensions were frozen in liquid N$_2$ and kept at -80°C before the XAS analyses.

**ICP MS measurement**

The Ce concentration was measured using ICP-MS (PerkinElmer, Nexlon 300X) in bacteria exposed to several CeO$_2$-4 nm concentrations: 0.01-0.1-1-5-10 mg CeO$_2$/L, from early- to late- exponential phases (t3). Bacterial pellets of three suspensions in 1 L-erlens were pooled to obtain sufficient material for the analysis (30 mg dry weight [DW]), no replicates were performed. Before ICP-MS analysis, each sample was dried and solubilized in acid using a Milestone UltraWAVE® microwave. The acids used for plant sample digestion were 2 mL HNO$_3$ and 1 mL H$_2$O$_2$. The measurement quality was controlled using certified reference materials (mussel tissue BCR-668 from IRMM). All Ce concentrations presented are expressed in mg per kg of dried matter.

**XAS measurements**

Ce speciation was measured by X-ray absorption near edge structure (XANES) spectroscopy at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The samples were analyzed in high energy resolution fluorescence detected mode (HERFD) on the FAME-UHD beamline (BM16) using a crystal analyzer spectrometer (CAS) equipped with 5 Ge(331) bent crystals (1 m radius of curvature). With CAS, photons are first optically selected in energy (Bragg diffraction) by the crystals and later collected by a detector, thereby generating a very small energy bandwidth (typically 1-2eV) and thus a higher detection limit and a better spectral resolution.$^{38,50}$ To avoid beam damage, the frozen pellets were analyzed at 10 K using a liquid He cryostat. No photoreduction effect was observed when comparing the different scans. All of the XANES spectra shown here were the sum of 2 to 12 scans, depending on the Ce concentration, in order to
improve the signal-to-noise ratio. Normalization and data reduction were performed using the IFFEFIT software package.\textsuperscript{51} Initial CeO\textsubscript{2}-NMs, Ce(III)-acetate, Ce(III)-oxalate, Ce(III)-phosphate and Ce(III)-cysteine were used as Ce(IV) and Ce(III) reference samples.

Quantitative determination of the Ce oxidation state was performed through a peak fitting analysis of the pre-edge features of the normalized spectra. To analyze the pre-edge features, a baseline subtraction was first performed by fitting a Gaussian function on the low energy tail of the edge using data a few eV before and after the pre-edge peak.\textsuperscript{52, 53} The pre-edges were then deconvoluted into Gaussian components (Pseudo-Voigt was also tested but did not improve the deconvolution performance). As a first step, the Gaussian parameters were allowed to vary for the standard compounds. The Gaussian parameters were then fixed and applied to all spectra (see for example Figure 2C for a deconvolution involving both Ce(III) and Ce(IV)). Two Gaussians functions were used to fit the Ce(III) contribution, with center max positions set at 5718.25 and 5720.87 eV and full widths at half maximum (FWHM) of 2.09 and 1.06 eV, respectively, while the area of each Gaussian function was set at 87% and 13% of the total area, respectively. The Ce(IV) contribution was fitted with one Gaussian with a center max position at 5720.12 eV and an FWHM of 1.77 eV. The pre-edge information was obtained by calculating the “integrated intensity” (sum of the integrated intensities of each component). We hypothesized that the intensity of the pre-edge peak reflects the proportion of the Ce(III) and Ce(IV), however this implies several assumptions. We put forward the hypotheses that: (i) there is a linear relation between the peak integrated area and the Ce oxidation state, (ii) that the local symmetry does not markedly influence the intensity and position of the pre-edge features. It would be difficult to conclude on this last point as the Ce(III) species formed in this study were not identified. To assess the uncertainty of the pre-edge fitting procedure, we calculated theoretical pre-edge spectra for mixtures of Ce(III) and Ce(IV) reference compounds (Ce(III)+Ce(IV)=100% and Ce(III) ranged from 5 to 95%). The peak fitting analysis described above was applied to these mixtures and the relative uncertainty was calculated. The uncertainty ranged from 3 to 22%. The results are thus all expressed with 22% uncertainty and presented as mean ± 0.22*mean.

Results and Discussion

Input of high-energy resolution fluorescence-detected X-ray absorption spectroscopy (HERFD-XAS) for the study of Ce speciation in highly diluted biological samples

Unequivocal identification of the Ce redox state in CeO\textsubscript{2} NMs

XANES at the Ce L3-edge was sensitive to the Ce oxidation state with one intense white line typical of Ce(III) (feature A in Ce(III)-acetate, Figure 1) and two peaks for Ce(IV) (features B and C in CeO\textsubscript{2} NMs, Figure 1) related to the Ce 5d electron density of states.\textsuperscript{40} However, feature A, located at 5729 eV, could also be attributed to crystal field splitting
(into $e_g$ and $t_{2g}$) for Ce(IV) in CeO$_2$ nanoparticles. With HERFD-XANES, the pre-edge features clearly distinguished the contributions of Ce(III) and Ce(IV) to the signal (Figure 2b). The Ce(III) compound pre-edges showed two features, i.e. D and F, with maximum peaks at 5718 and 5721 eV, respectively, while the Ce(IV) compound pre-edges showed a single peak E with a maximum at 5720 eV. Peaks D and E were easily distinguishable with HERFD-XAS as they were separated by approx. 2eV. Regardless of the NM size, HERFD-XANES analysis solely revealed the presence of the Ce(IV) oxidation state (SI Table 2) in the CeO$_2$ NMs tested before interaction with bacteria. This result is in agreement with the previously observed absence of Ce(III) inside CeO$_2$ NMs.

**Major advance in the speciation of highly diluted Ce bacteria**

In this study, we analyzed Ce in bacteria exposed to concentrations as low as 10 µg Ce/L. XAS measurement using CAS allows the study of diluted chemical elements and therefore realistic NM concentrations. The predicted Ce concentration in soil ranged from ng/kg to mg/kg, depending on the area and the soil usage, such as sludge-amended soil or urban soil$^{32,36,55}$, but no predictions of environmental Ce concentrations in soil solution have been published to date. In aquatic media, the Ce predicted environmental concentration ranged from 1 ng/L to 1 µg/L$^{36}$ With bacterial cell concentrations ranging from $10^4$ to $10^6$ CFU/mL, thereby representing an NM/cell ratio ranging from 4.8 to 4.8 $10^5$. In this study, the bacterial density ranged from 4. $10^7$ to 2.8 $10^8$ CFU/mL, and the NM/cell ratio from 1.7 $10^2$ to 1.2 $10^6$ depending on the CeO$_2$ NM concentration exposure. Therefore, the NM/cell ratio tested in this study was very plausible compared to the environmental prediction.

The Ce L3-edge XANES of bacteria exposed to CeO$_2$-4nm ranged from 0.01 to 10 mg/L, indicating a dominant single absorption edge peak located at 5729 eV, which is typical of Ce(III) (Figure 3). The pre-edge peak analysis confirmed a major Ce(III) contribution of >70 ± 15% for 5 and 10 mg CeO$_2$/L concentrations (Table SI 3). The Ce concentrations of these bacteria ranged from 6.2 to 1870 mg Ce/kg DW (Table 1). To date, the lowest Ce concentration analyzed by bulk Ce L3 edge XANES in environmental samples was 35 mg Ce/kg DW.$^{11}$ HERFD-XANES analysis therefore improved the detection limit, since XANES were recorded for concentrations as low as 6.2 mg Ce/kg DW. To our knowledge, this is the first time that Ce speciation has been detected at concentrations below 10 mg/kg DW in biological samples. Note that the bacterial samples were analyzed frozen and hydrated (> 90 % water content), therefore with lower exposure Ce concentrations of around 1 mg/kg dried or freeze-dried samples could be used for the analyses.

**Size-dependent biotransformation of Ce NMs**

The bacterial growth cycle comprises three main phases, each with a specific physiological profile. In the lag phase, just after introduction in the fresh medium, cells sense the environment and retool the machinery in preparation for the exponential phase,
during which they actively divide, depending on the energy sources and nutrients. At the late-exponential phase, as the energy sources are depleted and metabolic waste products accumulate, cells enter in the stationary phase, where growth apparently ceases, with equivalent rates of bacterial cell growth and death. The late exponential phase is an interesting step in the bacterial growth cycle. Secondary metabolism involved in microbial and ecological interactions tends to occur at these late exponential and stationary bacterial growth phases.66

The speciation of the Ce accumulated in bacteria after exposure to three different sizes of CeO$_2$ NMs was measured in the late-exponential phase (Figure 1). We obtained contrasted results between bacteria exposed to 31 nm-spherical CeO$_2$ NMs and to the two small sizes: 4 nm-spherical and 7 nm-ellipsoidal. XANES spectra of bacteria exposed to 31 nm CeO$_2$ NMs overlapped the spectra of initial CeO$_2$ NMs, indicating that the atomic structure of the CeO$_2$ NMs was not markedly affected. XANES spectra of bacteria exposed to CeO$_2$-31 nm were fitted with 100% Ce(IV) using Ref 31 nm_CeO$_2$ (see Table SI 2). XANES spectra of bacteria exposed to 4 nm and 7 nm showed a dominant absorption edge (feature A, Figure 1) typical of the presence of Ce(III). The reduction was similar between the two sizes: pre-edge peak fitting indicated a contribution of Ce(III) of 70 ± 15% for 4 nm CeO$_2$ NMs and 64 ± 14% for 7 nm CeO$_2$ NMs at 5 mg Ce/L (Table SI 3). To avoid a potential Ce reduction due to the composition of the culture medium with 1.4 mM K$_2$HPO$_4$, CeO$_2$-4 nm was incubated in sterilized TSB/10 medium for 24 h and no Ce reduction was detected by XAS (Figure 4B). Our results showed that the 4 and 7 nm CeO$_2$ NMs were biotransformed whereas 31 nm were not modified after bacterial exposure.

Several physicochemical mechanisms could explain the differences in Ce reduction for different CeO$_2$ NMs, such as aggregation, specific surface area and reactivity.1,8,28,57 The aggregation state in the media could influence the amount of surface-exposed NMs to bacterial cells. Yan et al. suggested that the reduction rates of iron oxide nanoparticles of different sizes (10, 30 and 50 nm) by Geobacter sulfurreducens was directly correlated with the bacteria-hematite contact area, while taking NM aggregation into account, not with the total oxide surface area.58 The isoelectric points (IEP) of CeO$_2$ NMs, and the pH and ionic strength of the culture media suggested that aggregation could also be expected for the three types of CeO$_2$ NMs. Since only the 4 and 7 nm CeO$_2$ NMs were bioreduced, no simple correlation was noted in this study between the aggregation state and the reduction rate.

The Ce(IV) reduction could also be attributed to the redox cycle occurring at the surface of the solid and/or to reductive dissolution.59 The percentage of surface atoms for the 31-nm NMs was around 5% (Table SI1), whereas the uncertainty of the HERDF-XAS method was ± 22%. This could explain why Ce(III) was not detected at the surface of 31 nm CeO$_2$ NMs. For the 4 nm and 7 nm CeO$_2$ NMs, the percentage of Ce(IV) reduction (> 64±14%, Table SI3) was higher than that of surface atoms (>36 %, Table SI1). We suggest that this reduction could be explained by reductive dissolution. Indeed, Ce(III) has high solubility,
with logKs = -2.15 for Ce(III) oxide (Smith, 2004), and its release rate could be expected to exponentially increase as the grain size decreases. To conclude, we hypothesized that the absence, or low Ce reduction, for the 31 nm NMs could be explained by a low reductive dissolution rate due to a lower percentage of surface atoms compared to NMs of under 7 nm.

**Biotransformation kinetics**

Metabolism is dependent on the phase of the growth cycle. Different sets of metabolites are produced in the primary metabolism, related to cell growth and division vs. the secondary metabolism, in the late-exponential and the stationary phases, related to ecological function. To determine the metabolic context in which Ce reduction takes place, we investigated the NM biotransformation kinetics by sampling cells after different exposure times to CeO$_2$-4 nm and CeO$_2$-7 nm, from the early-exponential to the stationary phase. Figure 2 shows that for each tested exposure time, a dominant post-absorption edge peak at 5729 eV, characteristic of Ce(III), was observed on all XANES, after exposure to CeO$_2$-7 nm. The pre-edge peak fitting analysis indicated similar results for all exposure times: 68 ± 15% Ce(III) after 45 and 90 min and 64 ± 14% Ce(III) after 320 min in bacteria exposed to 7 nm CeO$_2$ (Table SI3). Similar results were obtained after exposure to CeO$_2$-4 nm: 76 ± 17% Ce(III) after 45 min and 70 ± 15% after 90 and 320 min (Table SI3). Therefore, these results indicated that a partial reduction occurred rapidly (in less than 45 min) in the early-exponential phase and did not vary up to the stationary phase. Interestingly, the conversion of glucose to gluconic acid and to 2-keto-gluconic acid by *Pseudomonas* occurred from the lag to the early exponential phases, which suggests a potential role of these metabolites in the reduction of CeO$_2$ NMs.

**Secretome and interaction between bacteria and NMs**

The secretome, i.e. the culture medium sampled after bacterial growth, including all the metabolites excreted by the bacteria, was isolated from the bacterial culture. Figure 4B shows XANES spectra of CeO$_2$-4 nm incubated in the bacterial secretome, sampled from a control or from a CeO$_2$-4 nm exposure sample. The spectra were similar between the NM suspension and after incubation in the two scenarios. The pre-edge analysis confirmed the absence of a Ce(III) contribution (Table SI3). The secretome therefore did not induce a Ce reduction after incubation with NMs. This indicates that the NM/bacterial interaction is needed to induce a Ce reduction. CeO$_2$ NMs can be adsorbed at the surface of the bacteria, as shown with *Escherischia coli* in the studies of Thill et al. and Zeyons et al. In the close vicinity of the bacteria, the chemical composition of the media was highly influenced by the bacterial exudation. Locally, the metabolite concentration was much higher than in the sampled secretome. Moreover, the metabolites could also be rapidly degraded in the media. Therefore, the bacterial metabolites in the secretome may not have induced a Ce reduction due to the low concentrations in the media and probable biodegradation.
Reduction of CeO$_2$ NMs is driven by 2-keto-gluconic acid and citric acid bacterial metabolites

To test the involvement of individual metabolites secreted in the extracellular medium in the Ce reduction, we focused on environmentally relevant metabolites that are known to be secreted in soil, especially in the rhizosphere, as *P. brassicacearum* NFN 421 is a good plant root colonizer.\(^{43}\) We selected 2-keto-gluconic acid and citric acid bacterial metabolites as potential sources of Ce reduction. As mentioned, these organic acids are produced by *Pseudomonas* sp., and more generally by Gram-negative bacteria, in their environment so as to retrieve phosphate, iron and interact with plants. *P. brassicacearum* contains all the genes necessary for the production and secretion of these metabolites.\(^{49}\) 2-keto-gluconic acid can be detected in the culture medium of *P. brassicacearum* in the early exponential phase (C. Santaella, personal communication).

CeO$_2$ NMs were incubated with 2-keto-gluconic acid, citric acid and ascorbic acid (which is not produced by bacteria), as ceria NMs can undergo a reduction with the assistance of these organic acids.\(^{11,17}\) All of the tested metabolites induced a partial reduction of CeO$_2$-4 nm after 24h exposure (Figure 4A). Peak fitting of the pre-edge confirmed the presence of Ce(III) in these samples with 23 ± 5% for citric acid, 30 ± 7% for 2-keto-gluconic acid and 26 ± 6% for ascorbic acid for CeO$_2$-4 nm, and 31 ± 7 % for ascorbic acid for CeO$_2$-7 nm (Table SI 4). Note that the bacterial metabolite 2-keto-gluconic acid proved to be as efficient as ascorbic acid, the main plant metabolite currently found to be involved in CeO$_2$ NMs reduction. As *Pseudomonas* species are ubiquitous bacteria that are frequently present in hydroponic plant cultures, and in the rhizosphere, this suggests the involvement of 2-keto-gluconic acid in CeO$_2$ NM reduction at the plant root interface. Around 36% of Ce atoms are localized at the surface of CeO$_2$-4 nm, and 41% for the CeO$_2$-7 nm. Assuming that the Ce reduction only occurred at the surface, the reductions induced by 2-keto-gluconic acid, citric acid and ascorbic acid for CeO$_2$-4 nm were 83 ± 19%, 65 ± 14% and 72 ± 17% of the surface atoms. For the CeO$_2$-7 nm, ascorbic acid induced a reduction of 75 ± 17% in the surface atoms. The metabolites alone, when present in excess in the suspension, were thus able to induce a marked reduction in CeO$_2$ NMs of the surface atoms. The reduction measured with the tested metabolites remained lower than in the biotic interaction. This could be explained by the implication of other metabolites not tested in this study and the effect of the colocalization of NMs and metabolites at the nanocell interface.

Overall, our results showed that the biotransformation and fate of NMs was size-dependent, and metabolites such as citric acid and 2-keto-gluconic acid proved efficient in reducing Ce from CeO$_2$-4 nm. Many soil bacteria other than *Pseudomonas* sp. can secrete 2-keto-gluconic acid,\(^{61}\) e.g. *Gluconobacter oxidans*. This suggests that in environmentally relevant conditions, the presence of soil microbiota associated with plant roots could contribute to the reduction of CeO$_2$ NMs. This biotransformation will affect the fate of Ce in the soil and the effect of Ce on soil biota (e.g. bacteria, fungi, macroorganisms and plants).
Beyond the fate of NMs in soil, this is an important result to take into consideration for diverse NM applications because the main properties of CeO$_2$ NMs will be impacted by biotransformation. For example, it is often speculated that CeO$_2$ NMs present an autoregenerative mechanisms of valence reversion, thereby explaining the catalytic properties. However, if NMs are transformed or if the reduction is total, as observed in our study, it is more than likely that Ce(III) would be released from CeO$_2$ NMs, thereby making the valence reversion impossible in NMs. Note that Pseudomonas sp. also include human pathogens such as *P. aeruginosa* or *P. putida*. Studies using CeO$_2$ NMs should therefore investigate possible contact of NMs with bacteria and adapt the NM size to take both the modification of NM properties and their environmental fate into account.

**Supporting Information:** Physicochemical characteristics of nanomaterials; Correlation between the optical density at 600 nm and colony forming units; *Pseudomonas brassicacearum* growth curve; Normalized Ce L$_3$-edge HERFD-XANES of CeO$_2$-4 nm, CeO$_2$-7 nm, CeO$_2$-31 nm and zoom on the pre-edge region; Pre-peak fitting parameters of the standard compounds; Results of the pre-peak fitting of the samples.

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Figures:

![Normalized Ce L3-edge high energy resolution fluorescence detected (HERFD) XANES of Pseudomonas brassicacearum exposed to three different sizes of nanomaterials (NMs): 4 nm, 7 nm and 31 nm CeO2 at 5 mg Ce.L^-1. Ce(III)–acetate, and the initial CeO2-NMs Ce(IV) are shown as reference compounds (BM13, ESRF). Bacteria were sampled at an optical density at 600 nm (OD600) of 0.7 (320 min exposure). Spectra of bacteria exposed to CeO2-7 nm are the same as the spectra t3 (OD600 0.7) 320 min in Figure 2. Feature A, located at 5729 eV, indicated a characteristic features of Ce(III) and features B and C characteristic features of Ce(IV). However, feature A, could also be attributed to crystal field splitting (into eg and t2g) for Ce(IV) in CeO2 NMs.](image-url)
Figure 2 – A) Normalized Ce L_3-edge high energy resolution fluorescence detected (HERFD) XANES of *Pseudomonas brassicacearum* exposed to CeO_2-7 nm at 5 mg Ce.L^{-1} for several incubation times: t1 (OD_{600} 0.15) 45 min, t2 (OD_{600} 0.3) 90 min, t3 (OD_{600} 0.7) 320 min. Ce(III)-acetate, and the CeO_2-7 nm are shown as reference compounds (BM13, ESRF). Spectra t3 (OD_{600} 0.7) are the same as the spectra shown in Figure 1 of bacteria exposed to CeO_2-7 nm. B) Zoom of the pre-edge region for the reference compound and *Pseudomonas brassicacearum* exposed to CeO_2-7 nm at 5 mg Ce.L^{-1} for 45 min t1 (OD_{600} 0.15). The Ce(III) compound pre-edges showed two features, i.e. D and F, with maximum peaks at 5718 and 5721 eV, respectively, while the Ce(IV) compound pre-edges showed a single peak E with a maximum at 5720 eV. C) Extracted pre-edge of *Pseudomonas brassicacearum* exposed to CeO_2-7 nm at 5 mg Ce.L^{-1} for 45 min t1 (OD_{600} 0.15) and the corresponding pre-edge peak fitting after baseline subtraction. Two Gaussian functions fitted the Ce(III) and one fitted the Ce(IV).
Figure 3 - Normalized Ce L3-edge high energy resolution fluorescence detected (HERFD) XANES of *Pseudomonas brassicacearum* exposed to CeO2-4 nm at several concentrations from 0.01 to 10 mg Ce L\(^{-1}\). Ce(III)–acetate, and the initial CeO\(_2\) NMs are shown as reference compounds (BM13, ESRF).
Figure 4 - Normalized Ce L₃-edge high energy resolution fluorescence detected (HERFD) XANES of CeO₂-4 nm and different incubations (A) incubations of CeO₂-4 nm with the growth culture medium (TSB1/10) and several metabolites: ascorbic acid, citric acid, 2-keto-gluconic acid (B) incubations of CeO₂-4 nm NMs with the secretome: culture medium (TSB1/10) after 24 h of *Pseudomonas brassicacearum* growth, exposed or not to CeO₂-4 nm during growth.
Table 1: Ce concentration (mg.kg\(^{-1}\) dry weigh) in bacteria *Pseudomonas brassicacearum* exposed at several CeO\(_2\)-4 nm concentrations from the early- to late- exponential phase nanoparticles OD\(_{600}\) 0.7 (2.8 \(10^8\) CFU/mL).

<table>
<thead>
<tr>
<th>CeO(_2) concentration in exposure medium (mg.L(^{-1}))</th>
<th>Ce concentration in bacteria (mg.kg(^{-1})DW)</th>
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