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Bacterial metabolites and particle size determine cerium oxide nanomaterial biotransformation

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3 1 **Bacterial metabolites and particle size determine cerium oxide nanomaterial**
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5 2 **biotransformation**
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11 51 **Abstract**

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15 52 Soil is a major receptor of manufactured nanomaterials (NMs) following unintentional
16 53 releases or intentional uses. Ceria NMs have been shown to undergo biotransformation in
17 54 plant and soil organisms with a partial Ce(IV) reduction into Ce(III) but the influence of
18 55 environmentally widespread soil bacteria is poorly understood. We used high-energy
19 56 resolution fluorescence detected X-ray absorption spectroscopy (HERFD-XAS) with an
20 57 unprecedented detection limit to assess Ce speciation in a model soil bacterium
21 58 (*Pseudomonas brassicacearum*) exposed to CeO₂ NMs of different sizes and shapes. The
22 59 findings revealed that the CeO₂ NMs size drives the biotransformation process. No
23 60 biotransformation was observed for the 31 nm CeO₂ NMs, contrary to 7 nm and 4 nm CeO₂
24 61 NMs, with a Ce reduction of $64 \pm 14\%$ and $70 \pm 15\%$, respectively. This major reduction
25 62 appeared quickly, from the early exponential bacterial growth phase. Environmentally
26 63 relevant organic acid metabolites secreted by *Pseudomonas*, especially in the rhizosphere,
27 64 were investigated. The 2-keto-gluconic and citric acid metabolites alone were able to
28 65 induce a significant reduction in 4 nm CeO₂ NMs. The high biotransformation measured
29 66 for < 7 nm NMs would affect the fate of Ce in the soil and biota.

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67 **Synopsis**

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

72 **Introduction**

74 The biological transformation of nanomaterials (NMs) is a key to understanding the
75 impact and fate of NMs in the environment.^{1,2} Soil is a major environmental collector for
76 NMs, along with sediment and landfill.^{3,4} Soil is also a complex ecological system hosting
77 a diverse range of living organisms and millions of microorganisms that can induce
78 environmental NM transformation at the scale of the organisms, tissues, cells and
79 biomolecules. Plants and microorganisms secrete secondary metabolites, i.e. specialized
80 molecules, that are essential for their biological activities while modulating their
81 interactions with the environment and neighboring organisms. Despite soil biodiversity,
82 NM biotransformation in terrestrial ecosystems has primarily focused on plant-
83 nanomaterial interfaces and several studies have assessed the fate of CeO₂ NMs in the
84 vicinity of plant roots.⁵⁻⁷ Due to the electronic structure of CeO₂ NMs, the Ce(IV)/Ce(III).

85 ratio at the NM surface is variable, while the core atoms are mainly in a Ce(IV) state.⁸
86 Partial Ce reduction of CeO₂ NMs was measured in several plant species such as tomato⁹,
87 wheat⁵ and cucumber¹⁰. Owing to this versatile redox reactivity, CeO₂ NM is a good
88 candidate to assess the NM biotransformation in soil.

89 The root surface^{6,9,11,12} and root-soil interface⁷ are the sites of interaction between plants
90 and NMs. Root metabolites excreted at these interfaces induce a reduction of CeO₂ NMs in
91 plant systems.^{6,13} Plenty of metabolites are candidates (saccharides, amino acids, organic
92 acid, phenols, etc.), yet only citric acid and ascorbic acid were shown to reduce CeO₂ NMs,
93 through incubation experiments simulating root exudates.¹¹
94 Soil and root associated bacteria also release a large set of secondary metabolites in their
95 environment for their nutrition (dissolution and uptake of iron, phosphate, etc.), and for
96 their communication with other microorganisms and plants. For instance, biodissolution
97 of Au NMs has been induced by the associated microbiome of a freshwater macrophyte,
98 despite the high chemical stability of Au NMs.¹⁴ The main driver of Au biodissolution is its
99 oxidation and complexation with a secondary metabolite, i.e. cyanide, emitted by
100 microorganisms.¹⁴ Regarding CeO₂ NMs, their transformation by bacteria has been little
101 addressed in the literature and the mechanism is poorly documented. Up to 50% of
102 reduced Ce occur in *Escherichia coli* and *Synechocystis* sp. exposed to 7 nm CeO₂ NMs.^{15,16}
103 However, the soil bacterium *Bacillus subtilis* has proven inefficient in reducing Ce in rod-
104 ceria 8.5 nm x.168 nm NMs, without the addition of exogenous ascorbic acid.¹⁷ The
105 bacterial cell surface is the nano-bio interface that promotes the reduction of CeO₂ NMs,¹⁶

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3 106 ¹⁷ however the mechanisms and the potential bacterial metabolites allowing this
4 107 reduction are still unknown.

6 108 What are the differences in terms of cell interface and secondary metabolism between
7 109 bacteria that do (*E. coli* and *Synechocystis* sp., gram-negative bacteria) or do not reduce
8 110 (*B. subtilis*, gram-positive bacteria) CeO₂ NMs? Apart from the structural differences in
9 111 their outer envelope, Gram-negative and Gram-positive bacteria differ in the way they
10 112 metabolize glucose, i.e. a carbon source for bacteria. Both take up glucose and metabolize
11 113 it by phosphorylation to generate energy-rich molecules (e.g. ATP). However, Gram-
12 114 negative bacteria can shut down this pathway and enable rapid conversion of glucose to
13 115 gluconic acid, and then to 2-keto-gluconic acid instead.^{18, 19} Gluconic acid and 2-keto-
14 116 gluconic acid can both be secreted at the outer bacterial membrane surface, and later
15 117 internalized to feed the cell metabolism. Gluconic acid is the preferred substrate, and is
16 118 metabolized to pyruvic acid and then citric acid through the Krebs cycle.²⁰ 2-keto-gluconic
17 119 acid is a reserve substrate for *Pseudomonas* sp. and known to accumulate in the
18 120 rhizosphere.²¹ This ability to rapidly convert and subtract glucose from other species,
19 121 and to secrete 2-keto-gluconic acid is a key feature involved in the ecological fitness of
20 122 Gram-negative bacteria, such as *Pseudomonas* sp., a diverse, ubiquitous and ecologically
21 123 significant soil bacteria. Citric acid and 2-keto-gluconic acid are important metabolites
22 124 secreted by *Pseudomonas* sp. to solubilize phosphate²² and chelate iron²³. As they are
23 125 released at the cell membrane interface, these two organic acids stand out as two
24 126 potential metabolite candidates for CeO₂ NM biotransformation.

25 127 Key physicochemical properties of NMs, such as their shape, size and surface charge, drive
26 128 their fate and their biological impact in the environment.²⁴ Among octahedral, cubic, rod,
27 129 and irregularly shaped CeO₂-NMs, rod-like particles are transformed to a greater degree
28 130 than other CeO₂-NMs in solutions simulating plant exudates in hydroponic cucumber
29 131 plants.²⁵ According to the particle shape and surface chemistry, CeO₂ NMs exhibits both
30 132 pro- and antioxidant activities, with the former being toxic to *E. coli*²⁶. The surface charge
31 133 of CeO₂ NMs has been shown to affect Ce(IV) biotransformation with a partial reduction
32 134 into Ce(III) in activated sludge bioreactors²⁷, in the nematode *Caenorhabditis elegans*²⁸,
33 135 and in wheat⁵ and tomato⁹. The size and aggregation of CeO₂ NMs drive the microbial
34 136 activity and bacterial community structure in the canola rhizosphere.²⁹ Therefore, the
35 137 biotransformation of CeO₂ NMs depends on the NM properties but also on the tested
36 138 organisms and environmental conditions.^{28, 30, 31} No marked relation between the size of
37 139 CeO₂ NMs and the extent of Ce reduction in the organisms has been noted to date.

38 140 While predicted concentration of CeO₂ NMs in soils ranges from ng.kg⁻¹ to mg.kg⁻¹ ³²⁻³⁶,
39 141 most studies addressing Ce biotransformation used concentrations close to or higher than
40 142 100 mg.kg⁻¹.³⁷ One of the reasons for this is the technical difficulty of detecting,
41 143 quantifying and characterizing CeO₂ NMs at lower concentrations in complex matrices.
42 144 Recent advances in X-ray absorption spectroscopy (XAS) such as high-energy resolution
43 145 fluorescence detected XAS (HERFD-XAS) have enabled measurement of XANES spectra
44 146 with higher energy resolution, i.e. with an apparent core hole lifetime broadening around
45 147 0.70eV, smaller than the probed electronic level bandwidth (around 3.2eV) and a lower

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3 148 detection limit (as low as 60 mg Ce/kg).^{38,39} The better defined edge and pre-edge
4 149 features in the HERFD-XAS spectra enable clear detection of the presence of Ce(III) in the
5 150 structure of CeO₂-NMs.^{39,40} It is essential to determine the oxidation state of CeO₂ NMs so
6 151 as to be able to identify the potential biotransformation of CeO₂ NMs, a process which
7 152 influences their bioavailability and potential ecotoxicity.^{13,41,42}
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11 154 This study aimed at identifying the biotransformation mechanisms of different sizes and
12 155 shapes of CeO₂ NMs by *P. brassicacearum* NFM421, a phytobenefic soil bacteria isolated
13 156 from *Arabidopsis thaliana* and *Brassica napus* plants.⁴³ We investigated the fate of CeO₂
14 157 NMs in contact with *P. brassicacearum* cells in planktonic conditions, and in cell-free
15 158 incubations with specific metabolites of citric acid and 2-keto-gluconic acid. Ascorbic acid
16 159 was also tested as a positive control to assess Ce reduction. The bacterial metabolism and
17 160 cell growth cycle are interconnected: different sets of metabolites are produced
18 161 throughout the three growth phases in planktonic conditions. We therefore explored CeO₂
19 162 NM reduction by *P. brassicacearum* at different steps of the growth cycle (i.e. the latence
20 163 phase, where cells adapt to their environment, the exponential phase, where cells divide,
21 164 and the stationary phase, where cells adapt to the nutrient reduction conditions), and we
22 165 tested the secretome, i.e. all metabolites excreted by the bacteria in the late exponential
23 166 phase. We used HERFD-XAS and were thus able to assess changes in Ce speciation and
24 167 identify the drivers of CeO₂ NMs biotransformation.
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33 170 **Materials and Methods**

34 171 35 172 **CeO₂ nanomaterials**

36 173 Three different sizes of commercially available types of bare CeO₂-NMs were used in this
37 174 study, all of which are made of cerianite crystallites. Transmission electron microscopy
38 175 indicated average sizes of 4 nm (CeO₂-4 nm) diameter with a spherical shape (Rhodia
39 176 Chemicals), 7 nm (CeO₂-7 nm) with an ellipsoidal shape (Rhodia Chemicals), and 31 nm
40 177 (CeO₂-31 nm) diameter with a spherical shape (Nanograin® Umicore). These NMs have
41 178 been previously described^{16,29,30,39,44-46} and their characteristics are summarized in the
42 179 Supporting Information (Table S1). The 7 nm NMs were an ellipsoid of revolution with an
43 180 average radius of the semi-axes of the ellipsoid of R = 3.5 and 0.91 nm. The specific surface
44 181 areas were estimated from their size and shape: 231 m².g⁻¹ for CeO₂-4 nm, 290 m².g⁻¹ for
45 182 CeO₂-7 nm and 30 m².g⁻¹ for CeO₂-31 nm. The surface atom percentages were calculated
46 183 using the formula given in Deshpande *et al.*⁴⁷ using different lattice parameters (0.557
47 184 nm 0.553 and 0.547 nm for 4, 7 and 31 nm CeO₂ NMs). We hypothesized that the outer
48 185 shell of atoms has a finite thickness of half the lattice constant.⁴⁷ The percentages of
49 186 surface atoms were approximately 36%, 41% and 5.2% for the 4 nm, 7 nm and 32 nm
50 187 forms, respectively).
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58 188 **Bacteria and incubation conditions.**

59 189 The *Pseudomonas brassicacearum* strain NFM421, a phytobenefic soil-bacteria, was

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3 190 selected for this study. The *Pseudomonas* genus has a robust metabolism for different
4 191 carbon sources, and a three-pronged metabolic system to metabolize glucose,¹⁸ a
5 192 bacterial carbon source that can be released in the rhizosphere from plant roots.⁴⁸ The *P.*
6 193 *brassicacearum* genome is sequenced, thereby allowing metabolic pathway prediction.⁴⁹
7
8 194 Bacterial growth involves three different main phases: i) a lag phase, i.e. a nonreplication
9 195 period during which the bacterial cells adapt; ii) an exponential phase, during which cell
10 196 division occurs at a constant rate, with a generation time close to 55 min for *P.*
11 197 *brassicacearum*; and iii) the stationary phase, where the growth rate levels off at zero,
12 198 while the bacteria stay metabolically active and compensate for those that die.

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16 199 *P. brassicacearum* NFM421 from frozen stock (-80°C) was grown at 30°C in 10-fold
17 200 diluted tryptic soy broth (TSB 1/10) solidified with 15 g L^{-1} agar (Sigma) under dark
18 201 conditions. After 48 h, a single colony was resuspended in TSB 1/10, incubated overnight
19 202 at 30°C and stirred at 150 rpm in an orbital shaker, until a concentration of 1×10^9 cells
20 203 per mL in stationary phase was reached, and this was used as a pre-culture.

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24 204 Aliquots of the pre-culture were transferred to a new TSB 1/10 medium in order to reach
25 205 an optical density at 600 nm (OD_{600}) of 0.05 ($\sim 2.10^6$ CFU/mL Figure SI 2). Bacteria and
26 206 TSB/10 medium were transferred into 50 mL tubes or 1 L erlens, stirred at 150 rpm,
27 207 incubated until the early exponential growth phase was reached at $\text{OD}_{600} = 0.1$ ($\sim 4.10^7$
28 208 CFU/mL), after approximately 60 min. Several exposures of the *P. brassicacearum* soil
29 209 bacteria to different sized and shaped CeO_2 NMs were then performed:

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32 210 1) Exposure to the three sizes of CeO_2 NMs: 4 nm, 7 nm and 31 nm at 5 mg CeO_2/L , from
33 211 the early- to the late-exponential phase (t3: OD_{600} 0.7 ($2.8 \cdot 10^8$ CFU/mL)). (See SI
34 212 Figure SI 3).
- 35 213 2) Exposure to several CeO_2 -4 nm concentrations: 0.01-0.1-1-5-10 mg CeO_2/L , from
36 214 the early- to the late-exponential phase (t3). Note that the tested concentration did
37 215 not affect the bacterial growth.
- 38 216 3) Exposure to CeO_2 -7 nm at 5 mg CeO_2/L with bacterial sampling at different times
39 217 during the exponential phase: t1) OD_{600} 0.15, after 45 min of contact, which was less
40 218 than the generation time (cell division) for *P. brassicacearum*, t2) OD_{600} 0.3, 90 min
41 219 mid-exponential phase and t3) OD_{600} 0.7, 165 min, late-exponential phase.

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46 220 For each size of CeO_2 NMs, a stock solution at 0.3 g Ce/L was prepared prior to bacterial
47 221 culture dosing. The bacterial culture was incubated at 30°C and stirred at 150 rpm. After
48 222 the bacterial growth phase, the suspensions were centrifuged at 1500 g at 4°C to separate
49 223 the bacteria and supernatant. The supernatants were discarded, and the bacterial pellets
50 224 were either immediately frozen in 5 mm diameter pellets in liquid N_2 and stored at -80°C
51 225 for XAS measurements, or freeze-dried for ICP-MS analysis.

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55 226 The effect of the culture medium, single metabolites and secretome (i.e. all metabolites
56 227 excreted by the bacteria exposed or not to CeO_2 NMs) on the Ce reduction were tested.
57 228 For these analyses, only CeO_2 -4 nm was tested due to time constraints for the Synchrotron
58 229 analyses and because the extent of bioreduction after bacterial exposure was similar for

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3 230 4 nm and 7 nm (Figure 1). The culture medium (TSB 1/10) and several commercialized
4 231 metabolites (2 keto-gluconic acid, ascorbic acid, citric acid from Sigma Aldrich) were
5 232 incubated with CeO₂-4 nm. The culture medium (TSB 1/10) and citric acid were also
6 233 incubated with CeO₂-7 nm. Metabolite solutions were prepared at 50 mM concentration,
7 234 and CeO₂ NMs at 300 mg Ce/L (2 mM) concentration. The metabolites were in excess
8 235 compared to CeO₂ NMs. Solutions were adjusted to pH=7.2. The suspensions were
9 236 incubated at 25°C under agitation for 24 h. After the incubation, droplets of the
10 237 suspensions were frozen in liquid N₂ and kept at -80°C before XAS analyses.

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15 238 To test the influence on the Ce reduction of the secretome, the bacterial culture
16 239 supernatant was incubated with CeO₂ NMs. We hypothesized that the bacteria could
17 240 respond to stress when exposed to NMs responsible for specialized metabolite excretion.
18 241 We compared two different secretomes: the supernatant of *P. brassicacearum* not
19 242 exposed to NMs, after a bacterial growth to an OD₆₀₀ of 0.7 (same conditions as explained
20 243 above), and the supernatant of *P. brassicaceae* exposed to 5 mg.L⁻¹ CeO₂-4 nm. After the
21 244 bacterial growth, the suspensions were centrifuged to separate the bacteria and
22 245 supernatant. The 4-nm CeO₂ NMs were added to these supernatants at 300 mg Ce/L
23 246 concentration and incubated under agitation for 24h. After the incubation, droplets of the
24 247 suspensions were frozen in liquid N₂ and kept at -80°C before the XAS analyses.

25 248 **ICP MS measurement**

26 249 The Ce concentration was measured using ICP-MS (PerkinElmer, NexIon 300X) in
27 250 bacteria exposed to several CeO₂-4 nm concentrations: 0.01-0.1-1-5-10 mg CeO₂/L, from
28 251 early- to late- exponential phases (t₃). Bacterial pellets of three suspensions in 1 L-erlens
29 252 were pooled to obtain sufficient material for the analysis (30 mg dry weight [DW]), no
30 253 replicates were performed. Before ICP-MS analysis, each sample was dried and
31 254 solubilized in acid using a Milestone UltraWAVE[®] microwave. The acids used for plant
32 255 sample digestion were 2 mL HNO₃ and 1 mL H₂O₂. The measurement quality was
33 256 controlled using certified reference materials (mussel tissue BCR-668 from IRMM). All Ce
34 257 concentrations presented are expressed in mg per kg of dried matter.

35 258 **XAS measurements**

36 259 Ce speciation was measured by X-ray absorption near edge structure (XANES)
37 260 spectroscopy at the European Synchrotron Radiation Facility (ESRF, Grenoble, France).
38 261 The samples were analyzed in high energy resolution fluorescence detected mode
39 262 (HERFD) on the FAME-UHD beamline (BM16) using a crystal analyzer spectrometer
40 263 (CAS) equipped with 5 Ge(331) bent crystals (1 m radius of curvature). With CAS, photons
41 264 are first optically selected in energy (Bragg diffraction) by the crystals and later collected
42 265 by a detector, thereby generating a very small energy bandwidth (typically 1-2eV) and
43 266 thus a higher detection limit and a better spectral resolution.^{38,50} To avoid beam damage,
44 267 the frozen pellets were analyzed at 10 K using a liquid He cryostat. No photoreduction
45 268 effect was observed when comparing the different scans. All of the XANES spectra shown
46 269 here were the sum of 2 to 12 scans, depending on the Ce concentration, in order to

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3 270 improve the signal-to-noise ratio. Normalization and data reduction were performed
4 271 using the IFFEFIT software package.⁵¹ Initial CeO₂-NMs, Ce(III)-acetate, Ce(III)-oxalate,
5 272 Ce(III)-phosphate and Ce(III)-cysteine were used as Ce(IV) and Ce(III) reference samples.

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8 273 Quantitative determination of the Ce oxidation state was performed through a peak fitting
9 274 analysis of the pre-edge features of the normalized spectra. To analyze the pre-edge
10 275 features, a baseline subtraction was first performed by fitting a Gaussian function on the
11 276 low energy tail of the edge using data a few eV before and after the pre-edge peak.^{52, 53}
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13 277 The pre-edges were then deconvoluted into Gaussian components (Pseudo-Voigt was also
14 278 tested but did not improve the deconvolution performance). As a first step, the Gaussian
15 279 parameters were allowed to vary for the standard compounds. The Gaussian parameters
16 280 were then fixed and applied to all spectra (see for example Figure 2C for a deconvolution
17 281 involving both Ce(III) and Ce(IV)). Two Gaussians functions were used to fit the Ce(III)
18 282 contribution, with center max positions set at 5718.25 and 5720.87 eV and full widths at
19 283 half maximum (FWHM) of 2.09 and 1.06 eV, respectively, while the area of each Gaussian
20 284 function was set at 87% and 13% of the total area, respectively. The Ce(IV) contribution
21 285 was fitted with one Gaussian with a center max position at 5720.12 eV and an FWHM of
22 286 1.77 eV. The pre-edge information was obtained by calculating the “integrated intensity”
23 287 (sum of the integrated intensities of each component). We hypothesized that the intensity
24 288 of the pre-edge peak reflects the proportion of the Ce(III) and Ce(IV), however this implies
25 289 several assumptions. We put forward the hypotheses that: (i) there is a linear relation
26 290 between the peak integrated area and the Ce oxidation state, (ii) that the local symmetry
27 291 does not markedly influence the intensity and position of the pre-edge features. It would
28 292 be difficult to conclude on this last point as the Ce(III) species formed in this study were
29 293 not identified. To assess the uncertainty of the pre-edge fitting procedure, we calculated
30 294 theoretical pre-edge spectra for mixtures of Ce(III) and Ce(IV) reference compounds
31 295 (Ce(III)+Ce(IV)=100% and Ce(III) ranged from 5 to 95%). The peak fitting analysis
32 296 described above was applied to these mixtures and the relative uncertainty was
33 297 calculated. The uncertainty ranged from 3 to 22%. The results are thus all expressed with
34 298 22% uncertainty and presented as mean ± 0.22*mean.

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45 46 300 **Results and Discussion**

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49 302 **Input of high-energy resolution fluorescence-detected X-ray absorption** 50 303 **spectroscopy (HERFD-XAS) for the study of Ce speciation in highly diluted** 51 304 **biological samples**

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54 306 **Unequivocal identification of the Ce redox state in CeO₂ NMs**

55 307 XANES at the Ce L3-edge was sensitive to the Ce oxidation state with one intense white
56 308 line typical of Ce(III) (feature A in Ce(III)-acetate, Figure 1) and two peaks for Ce(IV)
57 308 (features B and C in CeO₂ NMs, Figure 1) related to the Ce 5d electron density of states.⁴⁰
58 309 However, feature A, located at 5729 eV, could also be attributed to crystal field splitting
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3 311 (into e_g and t_{2g}) for Ce(IV) in CeO₂ nanoparticles.³⁸ With HERFD-XANES, the pre-edge
4 312 features clearly distinguished the contributions of Ce(III) and Ce(IV) to the signal (Figure
5 313 2b). The Ce(III) compound pre-edges showed two features, i.e. D and F, with maximum
6 314 peaks at 5718 and 5721 eV, respectively, while the Ce(IV) compound pre-edges showed a
7 315 single peak E with a maximum at 5720 eV. Peaks D and E were easily distinguishable with
8 316 HERFD-XAS as they were separated by approx. 2eV. Regardless of the NM size, HERFD-
9 317 XANES analysis solely revealed the presence of the Ce(IV) oxidation state (SI Table 2) in
10 318 the CeO₂ NMs tested before interaction with bacteria. This result is in agreement with the
11 319 previously observed absence of Ce(III) inside CeO₂ NMs.^{40, 54} Therefore we confirmed the
12 320 absence of Ce(III) for several sizes of CeO₂ NMs.

321 322 **Major advance in the speciation of highly diluted Ce bacteria**

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324 In this study, we analyzed Ce in bacteria exposed to concentrations as low as 10 µg Ce/L.
325 XAS measurement using CAS allows the study of diluted chemical elements and therefore
326 realistic NM concentrations. The predicted Ce concentration in soil ranged from ng/kg to
327 mg/kg, depending on the area and the soil usage, such as sludge-amended soil or urban
328 soil^{32, 36, 55}, but no predictions of environmental Ce concentrations in soil solution have
329 been published to date. In aquatic media, the Ce predicted environmental concentration
330 ranged from 1 ng/L to 1 µg/L.³⁶ With bacterial cell concentrations ranging from 10⁴ to 10⁶
331 CFU/mL, thereby representing an NM/cell ratio ranging from 4.8 to 4.8 10⁵. In this study,
332 the bacterial density ranged from 4. 10⁷ to 2.8 10⁸ CFU/mL, and the NM/cell ratio from
333 1.7 10² to 1.2 10⁶ depending on the CeO₂ NM concentration exposure. Therefore, the
334 NM/cell ratio tested in this study was very plausible compared to the environmental
335 prediction.

336
337 The Ce L3-edge XANES of bacteria exposed to CeO₂-4nm ranged from 0.01 to 10 mg/L,
338 indicating a dominant single absorption edge peak located at 5729 eV, which is typical of
339 Ce(III) (Figure 3). The pre-edge peak analysis confirmed a major Ce(III) contribution of >
340 70 ± 15% for 5 and 10 mg CeO₂/L concentrations (Table SI 3). The Ce concentrations of
341 these bacteria ranged from 6.2 to 1870 mg Ce/kg DW (Table 1). To date, the lowest Ce
342 concentration analyzed by bulk Ce L3 edge XANES in environmental samples was 35 mg
343 Ce/kg DW.¹¹ HERFD-XANES analysis therefore improved the detection limit, since XANES
344 were recorded for concentrations as low as 6.2 mg Ce/kg DW. To our knowledge, this is
345 the first time that Ce speciation has been detected at concentrations below 10 mg/kg DW
346 in biological samples. Note that the bacterial samples were analyzed frozen and hydrated
347 (> 90 % water content), therefore with lower exposure Ce concentrations of around 1
348 mg/kg dried or freeze-dried samples could be used for the analyses.

349 350 **Size-dependent biotransformation of Ce NMs**

351 The bacterial growth cycle comprises three main phases, each with a specific
352 physiological profile. In the lag phase, just after introduction in the fresh medium, cells
353 sense the environment and retool the machinery in preparation for the exponential phase,

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3 354 during which they actively divide, depending on the energy sources and nutrients. At the
4 355 late-exponential phase, as the energy sources are depleted and metabolic waste products
5 356 accumulate, cells enter in the stationary phase, where growth apparently ceases, with
6 357 equivalent rates of bacterial cell growth and death. The late exponential phase is an
7 358 interesting step in the bacterial growth cycle. Secondary metabolism involved in
8 359 microbial and ecological interactions tends to occur at these late exponential and
9 360 stationary bacterial growth phases.⁵⁶
10 361

11 362 The speciation of the Ce accumulated in bacteria after exposure to three different sizes of
12 363 CeO₂ NMs was measured in the late-exponential phase (Figure 1). We obtained contrasted
13 364 results between bacteria exposed to 31 nm-spherical CeO₂ NMs and to the two small sizes:
14 365 4 nm-spherical and 7 nm-ellipsoidal. XANES spectra of bacteria exposed to 31 nm CeO₂
15 366 NMs overlapped the spectra of initial CeO₂ NMs, indicating that the atomic structure of
16 367 the CeO₂ NMs was not markedly affected. XANES spectra of bacteria exposed to CeO₂-31
17 368 nm were fitted with 100% Ce(IV) using Ref 31 nm_CeO₂ (see Table SI 2). XANES spectra
18 369 of bacteria exposed to 4 nm and 7 nm showed a dominant absorption edge (feature A,
19 370 Figure 1) typical of the presence of Ce(III). The reduction was similar between the two
20 371 sizes: pre-edge peak fitting indicated a contribution of Ce(III) of 70 ± 15% for 4 nm CeO₂
21 372 NMs and 64 ± 14% for 7 nm CeO₂ NMs at 5 mg Ce/L (Table SI 3). To avoid a potential Ce
22 373 reduction due to the composition of the culture medium with 1.4 mM K₂HPO₄, CeO₂-4 nm
23 374 was incubated in sterilized TSB/10 medium for 24 h and no Ce reduction was detected by
24 375 XAS (Figure 4B). Our results showed that the 4 and 7 nm CeO₂ NMs were biotransformed
25 376 whereas 31 nm were not modified after bacterial exposure.
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27 378 Several physicochemical mechanisms could explain the differences in Ce reduction for
28 379 different CeO₂ NMs, such as aggregation, specific surface area and reactivity.^{1, 8, 28, 57} The
29 380 aggregation state in the media could influence the amount of surface-exposed NMs to
30 381 bacterial cells. Yan *et al.* suggested that the reduction rates of iron oxide nanoparticles of
31 382 different sizes (10, 30 and 50 nm) by *Geobacter sulfurreducens* was directly correlated
32 383 with the bacteria-hematite contact area, while taking NM aggregation into account, not
33 384 with the total oxide surface area.⁵⁸ The isoelectric points (IEP) of CeO₂ NMs, and the pH
34 385 and ionic strength of the culture media suggested that aggregation could also be expected
35 386 for the three types of CeO₂ NMs. Since only the 4 and 7 nm CeO₂ NMs were bio-reduced, no
36 387 simple correlation was noted in this study between the aggregation state and the
37 388 reduction rate.
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39 390 The Ce(IV) reduction could also be attributed to the redox cycle occurring at the surface
40 391 of the solid and/or to reductive dissolution.⁵⁹ The percentage of surface atoms for the 31-
41 392 nm NMs was around 5% (Table SI1), whereas the uncertainty of the HERDF-XAS method
42 393 was ± 22%. This could explain why Ce(III) was not detected at the surface of 31 nm CeO₂
43 394 NMs. For the 4 nm and 7 nm CeO₂ NMs, the percentage of Ce(IV) reduction (> 64±14%,
44 395 Table SI3) was higher than that of surface atoms (>36 %, Table SI1). We suggest that this
45 396 reduction could be explained by reductive dissolution. Indeed, Ce(III) has high solubility,

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3 397 with logKs = -21.5 for Ce(III) oxide (Smith, 2004), and its release rate could be expected
4 398 to exponentially increase as the grain size decreases.^{31, 60} To conclude, we hypothesized
5 399 that the absence, or low Ce reduction, for the 31 nm NMs could be explained by a low
6 400 reductive dissolution rate due to a lower percentage of surface atoms compared to NMs
7 401 of under 7 nm.
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10 402 11 403 **Biotransformation kinetics**

12 404 Metabolism is dependent on the phase of the growth cycle. Different sets of metabolites
13 405 are produced in the primary metabolism, related to cell growth and division vs. the
14 406 secondary metabolism, in the late-exponential and the stationary phases, related to
15 407 ecological function.⁵⁶ To determine the metabolic context in which Ce reduction takes
16 408 place, we investigated the NM biotransformation kinetics by sampling cells after different
17 409 exposure times to CeO₂-4 nm and CeO₂-7 nm, from the early-exponential to the stationary
18 410 phase. Figure 2 shows that for each tested exposure time, a dominant post-absorption
19 411 edge peak at 5729 eV, characteristic of Ce(III), was observed on all XANES, after exposure
20 412 to CeO₂-7 nm. The pre-edge peak fitting analysis indicated similar results for all exposure
21 413 times: 68 ± 15% Ce(III) after 45 and 90 min and 64 ± 14% Ce(III) after 320 min in bacteria
22 414 exposed to 7 nm CeO₂ (Table SI3). Similar results were obtained after exposure to CeO₂-
23 415 4 nm: 76 ± 17% Ce(III) after 45 min and 70 ± 15% after 90 and 320 min (Table SI3).

24 416 Therefore, these results indicated that a partial reduction occurred rapidly (in less than
25 417 45 min) in the early-exponential phase and did not vary up to the stationary phase.
26 418 Interestingly, the conversion of glucose to gluconic acid and to 2-keto-gluconic acid by
27 419 *Pseudomonas* occurred from the lag to the early exponential phases, which suggests a
28 420 potential role of these metabolites in the reduction of CeO₂ NMs.
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37 422 **Secretome and interaction between bacteria and NMs**

38 423 The secretome, i.e. the culture medium sampled after bacterial growth, including all the
39 424 metabolites excreted by the bacteria, was isolated from the bacterial culture. Figure 4B
40 425 shows XANES spectra of CeO₂-4 nm incubated in the bacterial secretome, sampled from a
41 426 control or from a CeO₂-4 nm exposure sample. The spectra were similar between the NM
42 427 suspension and after incubation in the two scenarios. The pre-edge analysis confirmed
43 428 the absence of a Ce(III) contribution (Table SI 3). The secretome therefore did not induce
44 429 a Ce reduction after incubation with NMs. This indicates that the NM/bacterial interaction
45 430 is needed to induce a Ce reduction. CeO₂ NMs can be adsorbed at the surface of the
46 431 bacteria, as shown with *Escherichia coli* in the studies of Thill et al.¹⁶ and Zeyons et al.¹⁵.
47 432 In the close vicinity of the bacteria, the chemical composition of the media was highly
48 433 influenced by the bacterial exudation. Locally, the metabolite concentration was much
49 434 higher than in the sampled secretome. Moreover, the metabolites could also be rapidly
50 435 degraded in the media. Therefore, the bacterial metabolites in the secretome may not
51 436 have induced a Ce reduction due to the low concentrations in the media and probable
52 437 biodegradation.
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439 Reduction of CeO₂ NMs is driven by 2-keto-gluconic acid and citric acid bacterial 440 metabolites

441 To test the involvement of individual metabolites secreted in the extracellular medium in
442 the Ce reduction, we focused on environmentally relevant metabolites that are known to
443 be secreted in soil, especially in the rhizosphere, as *P. brassicacearum* NFN 421 is a good
444 plant root colonizer.⁴³ We selected 2-keto-gluconic acid and citric acid bacterial
445 metabolites as potential sources of Ce reduction. As mentioned, these organic acids are
446 produced by *Pseudomonas* sp., and more generally by Gram-negative bacteria, in their
447 environment so as to retrieve phosphate, iron and interact with plants. *P. brassicacearum*
448 contains all the genes necessary for the production and secretion of these metabolites⁴⁹.
449 2-keto-gluconic acid can be detected in the culture medium of *P. brassicacearum* in the
450 early exponential phase (C. Santaella, personal communication).

451 CeO₂ NMs were incubated with 2-keto-gluconic acid, citric acid and ascorbic acid (which
452 is not produced by bacteria), as ceria NMs can undergo a reduction with the assistance of
453 these organic acids.^{11, 17} All of the tested metabolites induced a partial reduction of CeO₂-
454 4 nm after 24h exposure (Figure 4A). Peak fitting of the pre-edge confirmed the presence
455 of Ce(III) in these samples with 23 ± 5% for citric acid, 30 ± 7% for 2-keto-gluconic acid
456 and 26 ± 6% for ascorbic acid for CeO₂-4 nm, and 31 ± 7 % for ascorbic acid for CeO₂-7
457 nm (Table SI 4). Note that the bacterial metabolite 2-keto-gluconic acid proved to be as
458 efficient as ascorbic acid, the main plant metabolite currently found to be involved in CeO₂
459 NMs reduction. As *Pseudomonas* species are ubiquitous bacteria that are frequently
460 present in hydroponic plant cultures, and in the rhizosphere, this suggests the
461 involvement of 2-keto-gluconic acid in CeO₂ NM reduction at the plant root interface.
462 Around 36% of Ce atoms are localized at the surface of CeO₂-4 nm, and 41% for the CeO₂-7
463 nm. Assuming that the Ce reduction only occurred at the surface, the reductions induced
464 by 2-keto-gluconic acid, citric acid and ascorbic acid for CeO₂-4 nm were 83 ± 19%, 65 ±
465 14% and 72 ± 17% of the surface atoms. For the CeO₂-7 nm, ascorbic acid induced a
466 reduction of 75 ± 17% in the surface atoms. The metabolites alone, when present in excess
467 in the suspension, were thus able to induce a marked reduction in CeO₂ NMs of the surface
468 atoms. The reduction measured with the tested metabolites remained lower than in the
469 biotic interaction. This could be explained by the implication of other metabolites not
470 tested in this study and the effect of the colocalization of NMs and metabolites at the nano-
471 cell interface.

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473 Overall, our results showed that the biotransformation and fate of NMs was size-
474 dependent, and metabolites such as citric acid and 2-keto-gluconic acid proved efficient
475 in reducing Ce from CeO₂-4 nm. Many soil bacteria other than *Pseudomonas* sp. can secrete
476 2-keto-gluconic acid,⁶¹ e.g. *Gluconobacter oxidans*. This suggests that in environmentally
477 relevant conditions, the presence of soil microbiota associated with plant roots could
478 contribute to the reduction of CeO₂ NMs. This biotransformation will affect the fate of Ce
479 in the soil and the effect of Ce on soil biota (e.g. bacteria, fungi, macroorganisms and
480 plants).

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3 481 Beyond the fate of NMs in soil, this is an important result to take into consideration for
4 482 diverse NM applications because the main properties of CeO₂ NMs will be impacted by
5 483 biotransformation. For example, it is often speculated that CeO₂ NMs present an
6 484 autoregenerative mechanisms of valence reversion, thereby explaining the catalytic
7 485 properties.⁴¹ Interesting, their ability to self-regenerate their surface has biomedical
8 486 applications.⁴² However, if NMs are transformed or if the reduction is total, as observed
9 487 in our study, it is more than likely that Ce(III) would be released from CeO₂ NMs, thereby
10 488 making the valence reversion impossible in NMs. Note that *Pseudomonas* sp. also include
11 489 human pathogens such as *P. aeruginosa* or *P. putida*. Studies using CeO₂ NMs should
12 490 therefore investigate possible contact of NMs with bacteria and adapt the NM size to take
13 491 both the modification of NM properties and their environmental fate into account.
14 492

15 493 **Supporting Information:** Physicochemical characteristics of nanomaterials; Correlation
16 494 between the optical density at 600 nm and colony forming units; *Pseudomonas*
17 495 *brassicacearum* growth curve; Normalized Ce L₃-edge HERFD-XANES of CeO₂-4 nm, CeO₂-
18 496 7 nm, CeO₂-31 nm and zoom on the pre-edge region; Pre-peak fitting parameters of the
19 497 standard compounds; Results of the pre-peak fitting of the samples.
20 498

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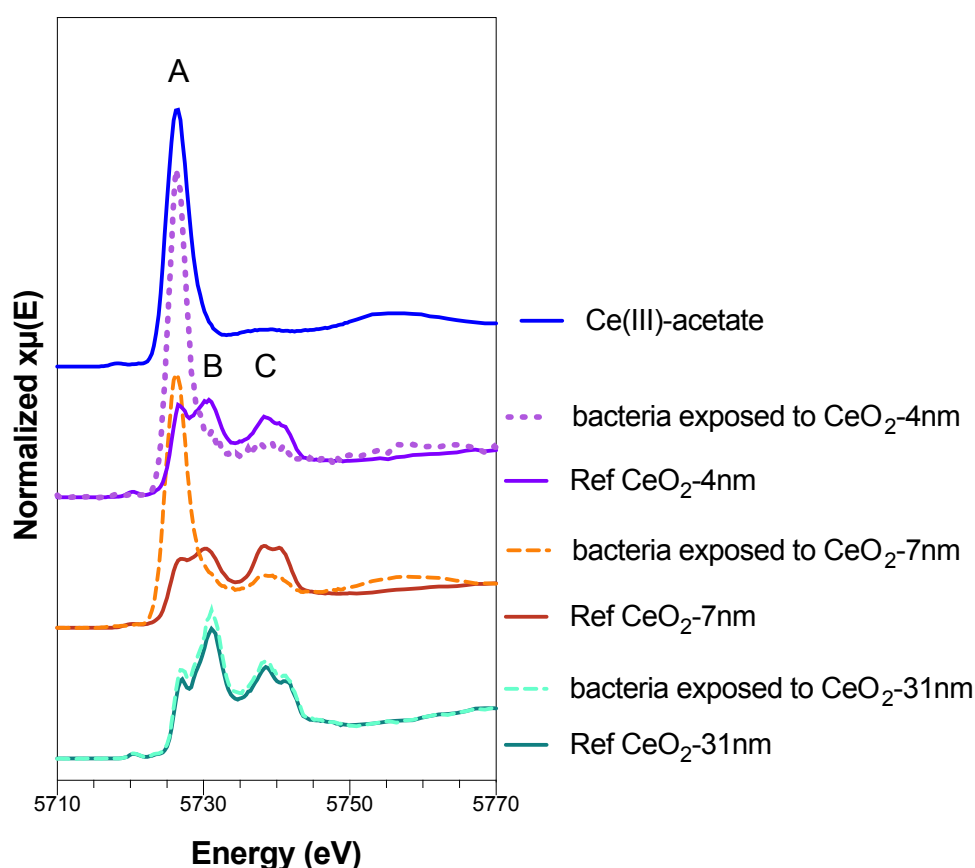
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16 710 **Figures :**

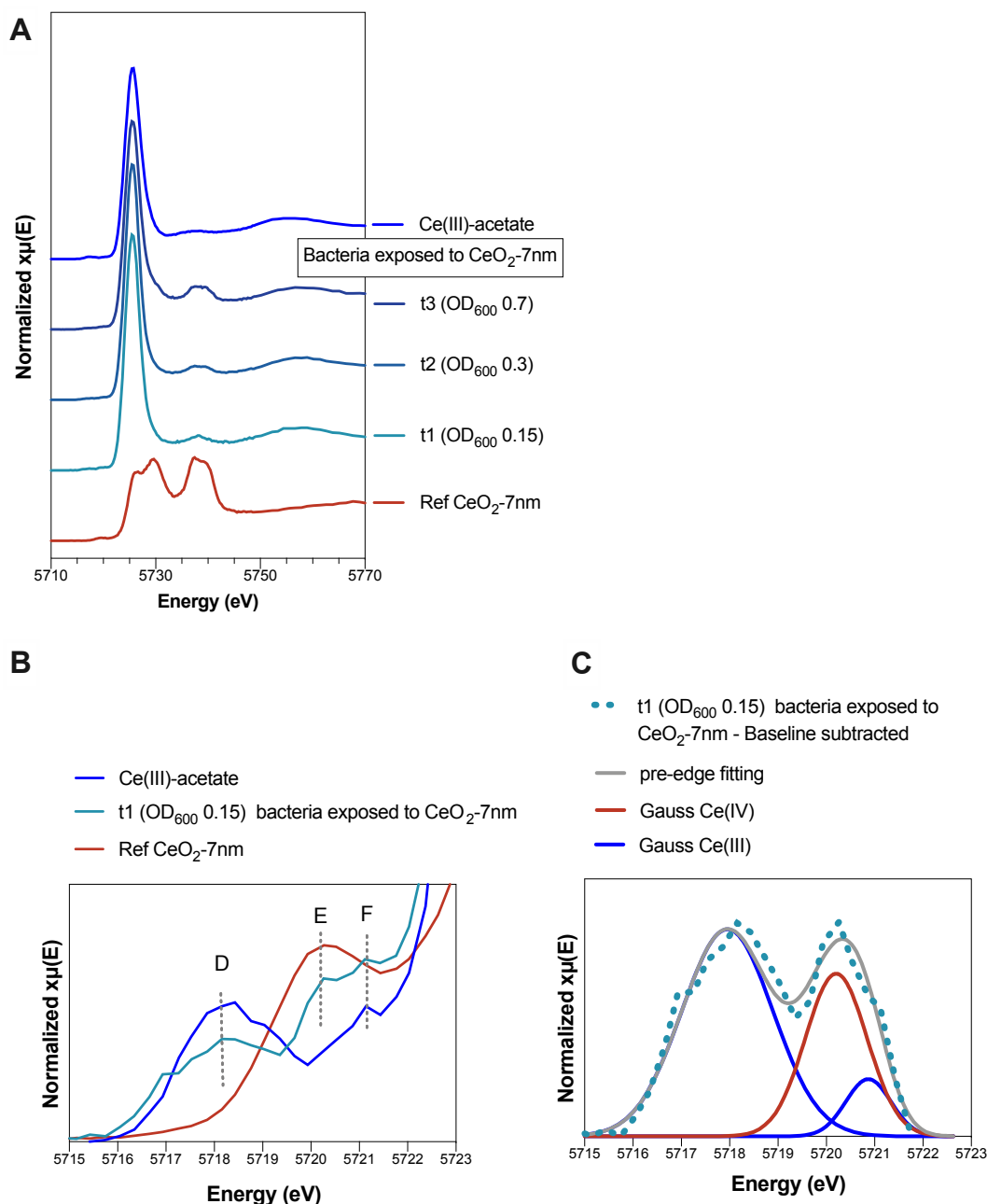


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47 713 **Figure 1 - Normalized Ce L₃-edge high energy resolution fluorescence detected (HERFD)**

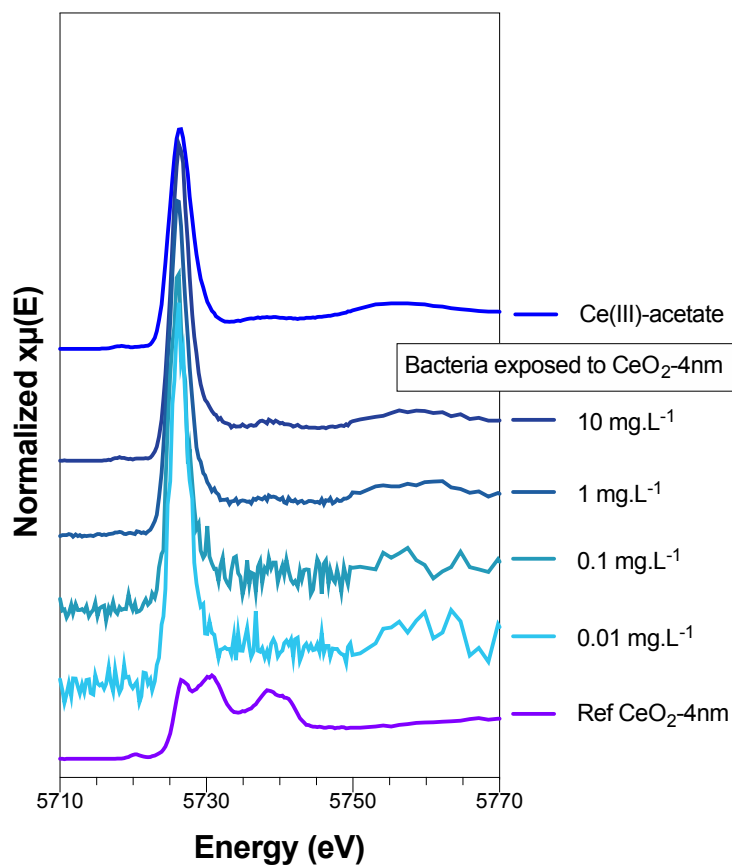
48 714 **XANES of *Pseudomonas brassicacearum* exposed to three different sizes of nanomaterials**
49 715 **(NMs): 4 nm, 7 nm and 31 nm CeO₂ at 5 mg Ce.L⁻¹. Ce(III)-acetate, and the initial CeO₂-NMs**
50 716 **Ce(IV) are shown as reference compounds (BM13, ESRF). Bacteria were sampled at an optical**
51 717 **density at 600 nm (OD₆₀₀) of 0.7 (320 min exposure). Spectra of bacteria exposed to CeO₂-7**
52 718 **nm are the same as the spectra t3 (OD₆₀₀ 0.7) 320 min in Figure 2. Feature A, located at 5729**
53 719 **eV, indicated a characteristic features of Ce(III) and features B and C characteristic features of**
54 720 **Ce(IV). However, feature A, could also be attributed to crystal field splitting (into eg and t_{2g})**
55 721 **for Ce(IV) in CeO₂ NMs.**
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724 **Figure 2 - A) Normalized Ce L_3 -edge high energy resolution fluorescence detected (HERFD)**
 725 **XANES of *Pseudomonas brassicacearum* exposed to CeO_2 -7 nm at 5 mg $Ce \cdot L^{-1}$ for several**
 726 **incubation times: t1 (OD_{600} 0.15) 45 min, t2 (OD_{600} 0.3) 90 min, t3 (OD_{600} 0.7) 320 min .**
 727 **Ce(III)-acetate, and the CeO_2 -7 nm are shown as reference compounds (BM13, ESRF). Spectra**
 728 **t3 (OD_{600} 0.7) are the same as the spectra shown in Figure 1 of bacteria exposed to CeO_2 -7 nm.**
 729 **B) Zoom of the pre-edge region for the reference compound and *Pseudomonas brassicacearum***
 730 **exposed to CeO_2 -7 nm at 5 mg $Ce \cdot L^{-1}$ for 45 min t1 (OD_{600} 0.15). The Ce(III) compound pre-edges**
 731 **showed two features, i.e. D and F, with maximum peaks at 5718 and 5721 eV, respectively, while**
 732 **the Ce(IV) compound pre-edges showed a single peak E with a maximum at 5720 eV. C)**
 733 **Extracted pre-edge of *Pseudomonas brassicacearum* exposed to CeO_2 -7 nm at 5 mg $Ce \cdot L^{-1}$ for 45**
 734 **min t1 (OD_{600} 0.15) and the corresponding pre-edge peak fitting after baseline subtraction.**
 735 **Two Gaussian functions fitted the Ce(III) and one fitted the Ce(IV).**



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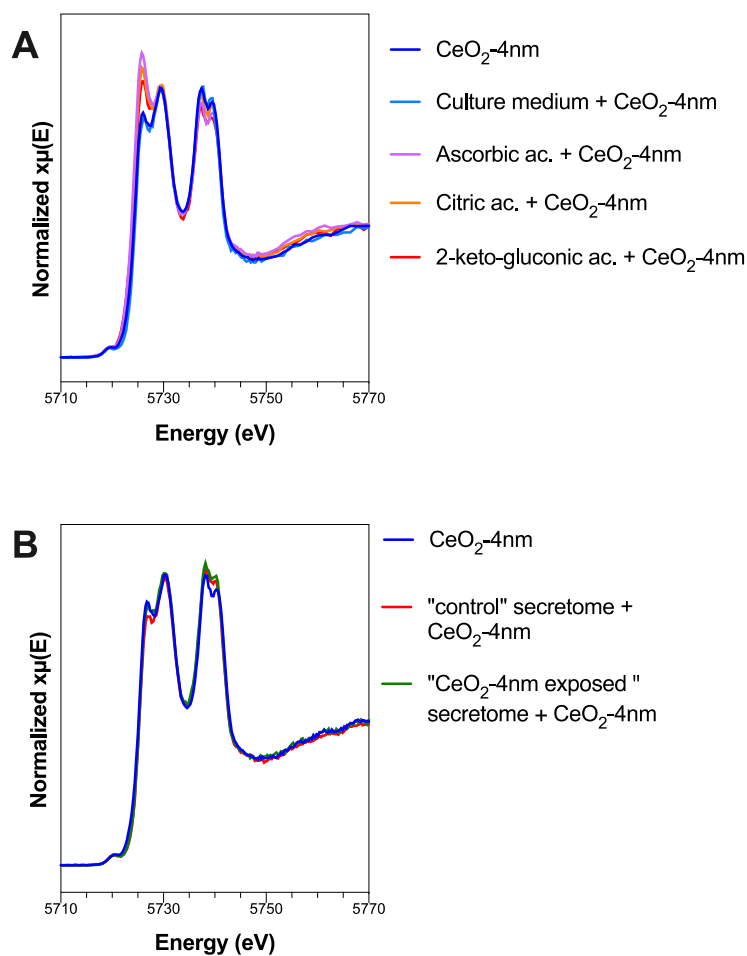
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Figure 3 - Normalized Ce L₃-edge high energy resolution fluorescence detected (HERFD) XANES of *Pseudomonas brassicacearum* exposed to CeO₂-4 nm at several concentrations from 0.01 to 10 mg Ce .L⁻¹. Ce(III)-acetate, and the initial CeO₂ NMs are shown as reference compounds (BM13, ESRF).



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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.

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Table 1: Ce concentration (mg.kg⁻¹ dry weigh) in bacteria *Pseudomonas brassicacearum* exposed at several CeO₂-4 nm concentrations from the early- to late- exponential phase nanoparticles OD₆₀₀ 0.7 (2.8 10⁸ CFU/mL).

CeO ₂ concentration in exposure medium (mg.L ⁻¹)	Ce concentration in bacteria (mg.kg ⁻¹ DW)
0.01	6.2
0.1	43
1	382
5	1196
10	1872

