Design Defines the Effects of Nanoceria at a Low Dose on Soil Microbiota and the Potentiation of Impacts by the Canola Plant

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Mohamed Hamidat[1,2], Mohamed Barakat[1,2], Philippe Ortet[1,2], Corinne Chanéac[2,3], Jérome Rose[2,4], Jean-Yves Bottero[2,4], Thierry Heulin[1,2], Wafa Achouak[1,2], and Catherine Santaella*[1,2]

1. Laboratory of Microbial Ecology of the Rhizosphere and Extreme Environments (LEMIRE), Aix-Marseille Université, CEA, CNRS, UMR 7265 Biosciences and biotechnology Institute of Aix-Marseille (BIAM), ECCOREV FR 3098, CEA Cadarache, St-Paul-lez-Durance, France
2. GDRi iCEINT, international Consortium for the Environmental Implication of Nanotechnology, CNRS-Duke University, F-13545 Aix-en-Provence, France
3. Chimie de la Matière Condensée, UMR7574, Collège de France, Université de Jussieu, Paris, F-75231, France
4. CNRS, Aix-Marseille Université, IRD, CEREGE UM34, F-13545 Aix en Provence, France

Author email addresses:
Mohamed Hamidat: hamidatmohamed@yahoo.fr
Mohamed Barakat: mohamed.barakat@cea.fr
Philippe Ortet: philippe.ortet@cea.fr
Corinne Chanéac: corinne.chaneac@upmc.fr
Jérome Rose: rose@cerege.fr
Jean-Yves Bottero: bottero@cerege.fr
Thierry Heulin: thierry.heulin@cea;fr
Wafa Achouak: wafa.achouak@cea.fr
Catherine Santaella: catherine.santaella@cea.fr
Corresponding author e-mail: catherine.santaella@cea.fr; tel: 033 442 257 713; Fax: 033 442 256 648

Abstract:

Soils act as nanoceria sinks *via* agricultural spreading and surface waters. Canola plants were grown for one month in soil spiked with nanoceria (1 $mg.kg^{-1}$). To define the role of nanomaterials design on environmental impacts, we studied nanoceria with different sizes (3.5 or 31 nm) and coating (citrate). We measured microbial activities involved in C, N and P cycling in the rhizosphere and unplanted soil. Bacterial community structure was analyzed in unplanted soil, rhizosphere and plant roots by 454-pyrosequencing of the 16S rRNA gene. This revealed an impact gradient dependent on nanomaterials design, ranging from decreased microbial enzymatic activities in planted soil to alterations in bacterial community structure in roots. Particle size/aggregation was a key parameter in modulating nanoceria effects on root communities. Citrate coating lowered the impact on microbial enzymatic activities but triggered variability in the bacterial community structure near the plant root. Some nanoceria favored taxa those closest relatives are hydrocarbon-degrading bacteria, and disadvantaged taxa frequently associated in consortia with disease-suppressive activity toward plant pathogens. This work provides a basis to determine outcomes of nanoceria in soil, at a dose close to predicted environmental concentrations, and to design them to minimize these impacts.

Introduction

Cerium oxide-based nanoparticles (nanoceria) have acquired great interest for their oxygen storage capacities, as well as their optical and catalytic properties. With a global estimated production of 10,000 metric tons/year.[1, 2] nanoceria are used in a variety of industrial sectors including fuel cells, electronic and optical devices, chemical mechanical polishing, exterior paints, and diesel fuel additives.[3] Some of these applications, such as coatings and paints, are dispersive during use phase. Modeling of the environmental exposure to engineered nanomaterials (ENMs) shows that soils are critical sinks for ENMs.[4] Keller and Lazareva estimated the global emission of nanoceria in soil to be in the range of 129-1029 metric tons/year.[5] This elevated value is certainly a concern for the potential risks of ENMs on soil.

Soil microbial communities provide critical ecosystem services including nutrient mineralization and cycling, and contribute to plant growth and health.[6] ENMs added to soil can have adverse effects on

microorganisms. In microcosms, nanoTiO_2 and nanoZnO (0.5-2 g.kg^{-1} soil) reduce microbial biomass and diversity and alter the composition of soil bacterial communities.[7] NanoTiO_2 transiently decreases C mineralization at 1 mg.kg^{-1} in silty soil rich in organic matter.[8] Nanosized particles of copper oxide (CuO) and magnetite (Fe_3O_4) (<50 nm, 1 g.kg^{-1}) change the hydrolytic activity and bacterial community composition of sandy clay loam soil[9] Colman et al. demonstrated the reduction of microbial extracellular enzymes and microbial biomass in soil treated with nanosilver (0.14 mg of Ag kg^{-1} soil) in a mesocosm study.[10] Recently, Ge et al. showed that nanoceria (100 mg.kg^{-1}) alter soil bacterial communities in soil planted with soybean,[11] although they do not affect soil bacterial communities in unplanted soils. The authors suggest that plants interactively promote nanoceria effects in soil, probably due to belowground carbon changes that result from toxic impact and plant growth decrease.

The interactions between plant roots and soil create a narrow zone of soil known as the rhizosphere, which is considered to be one of the most dynamic interfaces on earth.[12] Numerous biogeochemical processes take place at the plant root-soil interface, which are driven by plant root activity and mediated by soil microorganisms. These processes regulate terrestrial carbon and other cycling elements that sustain plant growth, as well as food, fuel and fiber production.[13] Understanding how ENMs can interact within the rhizosphere to influence plant and microbial community function and structure is thus crucial for a variety of ecosystem level processes. However, current knowledge on the impact of ENMs on plants and the rhizosphere microbiota is limited.

To date, research on the environmental impact of ENMs on soil has largely focused on high concentrations of nanomaterials; by contrast, soil environmental concentrations of nanoceria are predicted[14, 15] within the range of 0.28-1.12 mg.kg^{-1} and could be even lower (0.09 to 5.1mg.kg^{-1}) as predicted in Denmark.[16] The current study therefore investigates the impact of nanoceria on a soil-plant-bacteria micro-ecosystem at a concentration of 1 mg.kg^{-1}, which is close to an environmentally relevant nanoceria concentration.

Previous studies have highlighted the influence of physicochemical factors such as geometry, coating and surface functional groups on the biological effects of nanoparticles.[17, 18] Nevertheless, the consequences of nanomaterials design still need to be examined in soil. In the case of nanoceria,

particle diameter is critical regarding surface defects, Ce^{3+}/Ce^{4+} ratio, and a series of properties such as O_2 storage, enzymatic-mimetic activities.[19] Here, we have evaluated the influence of nanomaterials design on soil bacterial microbiota. The design criteria were focused on particle size and surface charge using pristine or citrate-coated nano-CeO_2, with different average particle diameters and surface coating.

The microbial activities involved in C, N and P recycling were examined in unplanted soil, and soil planted with canola after one month of exposure to nanoceria. Microbial community structure was analyzed by 454 pyrosequencing of the *rrs* gene encoding 16SrRNA in planted and unplanted soils, and in plant root systems. Our results reveal a range of gradual impacts dependent on nanomaterials design, from decreased microbial enzymatic activities to alterations in bacterial community structure. More importantly, some nanoceria could exert a selection pressure favoring taxa those closest relatives are aromatic hydrocarbon-degraders, more likely to be resistant to heavy metals and antibiotics, as well as disadvantaged taxa often identified in consortia associated with soil suppressiveness toward plant pathogens. Our results thus provide a basis to determine the potential outcomes of nanoceria in soil, and to design them so as to minimize these impacts, at realistic environmental concentration.

Materials and Methods

Characteristics of CeO_2 nanoparticles

Three different types of commercially available CeO_2 nanoparticles (NPs) were added to the soil. Two types were commercial crystallites of cerianite, with sizes of 31±18 nm, as measured by transmission electron microscopy (TEM) (CeO_2-U; Nanograin® Umicore), and 3.5±0.5 nm (for CeO_2-R). Citrate-coated crystallites of CeO_2-R are used as UV-stabilizer and have a TEM size of 3.9±1.8 nm (coated CeO_2; Nanobyk®-3810, Byk). These NPs have been previously described[20-22] and their characteristics are summarized in Supporting Information (SI) Table S1. CeO_2 NP suspensions were prepared in ultrapure water (UPW; Milli-Q®, Millipore). The compared colloidal behavior of CeO_2-NPs in a natural mineral water (pH 7.9), in interaction with clays (kaolinite), and the same natural water in aquatic mesocosms (organic carbon 2.0 ± 0.1 $mg.L^{-1}$) is fully described.[20-22] Briefly, pristine CeO_2-U tends to rapidly homo-aggregate in UPW (Table S1) and in mineral water (with or without clays),[22]

and is referred to as pristine CeO_2 aggregate in the text. Homo-aggregation in conjunction with hetero-aggregation with clays was observed for pristine CeO_2-R. Citrate-coated CeO_2-NPs homo- and hetero-aggregated but this required time-dependent degradation of the coating.

Soil

Topsoil (0–20 cm) from a clay-loam calcareous soil (pH 8.19) was collected from an agricultural parcel used for wheat culture in Aix-les-Platanes (France; 43°33'45.58'' N; 05°28'38.78'' E). The soil was sieved to 4 mm and stored at room temperature before use. Water pH of the soil was 8.19, with 4.3% organic matter, 152 mequiv.kg^{-1} cation exchange capacity, 27.1% sand, 21.5% silt, and 18.9% clay (Gammsol; InVivo Labs, France). Additional soil characteristics are summarized in Table S2.

Briefly, 1 kg of soil was deposited in a thin layer in a plastic bag. A suspension of CeO_2-NPs was distributed on the surface to achieve 1.0 mg.kg^{-1} dry soil mass and a humidity of 15% (w/w). The control soil was treated identically, but without the addition of NPs. The bag was closed and the soil was homogenized by repeatedly inverting the bag. Soil was sieved again to 4 mm. Sterile syringes (60 mL) were used as pots and filled with 70 g of soil. Three replicates were prepared for each treatment (control, CeO_2-R, CeO_2-U, and coated CeO_2).

Plant growth

Canola seeds were planted and plants were grown in phytotrons (SI). Plants were harvested after 30 days of growth. Aboveground plant parts were cut and weighed (fresh weight), dried at 60°C for three days, and weighed again (dried weight). The root system was retrieved from planted pots and washed repeatedly with sterile UPW. Excess water was blotted on sterile tissue paper and the root system was frozen in liquid N_2 and stored at -80°C or alternatively dried at 60°C for three days (dry root mass). The entirety of soil from planted pots was considered as rhizosphere,[11] which was manually cleared from root material, collected, frozen in liquid N_2 and stored at -80°C. Soil from unplanted pots, considered as bulk soil, was collected in the same way.

Enzymatic activity of soil and catalase mimetic activity of cerium oxide nanoparticles

Soil enzyme activities were determined for fresh collected materials. All enzyme activities were performed in triplicate for each treatment. Enzymatic activities are reported as the mean of the three determinations expressed in unit activity per g of dry soil mass. (SI).

Catalase mimetic activity of nanoceria was measured by the decrease in the absorbance of H_2O_2 at 240 nm, using a UV–visible spectrophotometer as described in Singh and Singh.[23]

DNA extraction and quantification and quantification

Total DNA was extracted from 0.5 g of soil or a root fraction using the FastDNA™ SPIN Kit for Soil and FastPrep®-24 Instrument (MP Biomedicals; Illkirch, France), according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry (NanoVue™-NV-GE, Healthcare Limited; UK), which measured the absorbance of the samples at 260-280 nm. As a sample control, fragments of the universal *rrs* gene (encoding 16S rRNA) were amplified by PCR using the universal bacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3'; positions 8-27 of the *rrs* gene from *E. coli*) and S17 (5'-GTTACCTTGTTACGACTT-3'; positions 1492-1509 of the *rrs* gene from *E. coli*). The products were analyzed by electrophoresis in 1.5% (w/v) agarose gels.

Total bacteria abundance was measured in the soil by quantitative PCR targeting of *rrs*. (SI).

Sequencing and post-run analysis

Barcoded amplicon sequencing processes (bTEFAP®) were performed by MR DNA **(www.mrdnalab.com**, MR DNA; Shallowater, TX**)** and are described in Dowd et al.[24] The *rrs* gene universal eubacterial primers 27Fmod (5'-AGRGTTTGATCMTGGCTCAG-3') and 530R (CCGCNGCNGCTGGCAC) were used to amplify the ~~rss~~ *rrs* gene regions V1–V3. Further details are provided in SI.

Sequence data resulting from the sequencing process were processed using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA; Shallowater, TX). Analysis of high-throughput community sequencing data was performed with QIIME version 1.8.[25] Further details are provided in SI. A total of 459,709 valid reads and 51,185 OTUs were obtained from the 36 samples through 454 pyrosequencing analysis. These OTUs were assigned to 29 different phyla. Each of the 36

communities contained between 3,285 and 35,089 reads, with OTUs ranging from 718 to 8,430 reads. The rarefaction curves (consisting of observed OTUs and the Chao1 estimator) tended to approach the saturation plateau for roots, but not for rhizosphere and bulk soil samples (Fig. 1a). Good's coverage estimations revealed that 86-95% of the species were obtained in root samples, whereas 82-88% of the species were determined in soil samples. Although no OTU-level rarefaction curves plateaued under the current sequencing depth, the Shannon diversity indices reached stable values (Figure S1b). This suggests that most of the microbial dominant diversity has been addressed in this data set.[26]

Statistical Analyses

The variables were checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). The significance of results was examined by one-way analysis of variance (ANOVA) followed by post-hoc tests (Student-Newman-Keul test). Pearson correlation coefficients were calculated to measure the strength of the association between microbial enzymatic activities. These analyses were performed in STATGRAPHICS Centurion XVI.II. $P<0.05$ was considered to be statistically significant.

For data that were not normally distributed, permutations (PERMANOVA, 999 permutations) and other non-parametric tests (non-parametric t-tests, Kruskal-Wallis test) were performed in R (http://www.R-project.org) or STATGRAPHICS. VEGAN's 'adonis' function was used to perform PERMANOVA with constrain permutations of nested factor ("strata" parameters). For multiple testing, P-values were corrected to Q-values using the Benjamini-Hochberg False Discovery Rate (FDR).[27] We selected a non-stringent Q-value set at 0.25 to avoid missing any important leads (http://www.biostathandbook.com/multiplecomparisons.html). Parametric and non-parametric statistics were implemented to identify OTU taxonomic groups (community modules) and individual OTUs (community members) that differ quantitatively between treatments within a compartment.[28] For analyses of OTU taxonomic groups, we prepared abundance matrices at the phylum rank containing the sum of OTU abundances for all OTUs per given taxon. Statistical comparisons of taxonomic groups were made for relative abundance using the Kruskal-Wallis test. OTU richness in soil is the result of a large number of low-count OTUs.[28] For individual OTU statistics, the follow-up analysis focused on the abundant community members (ACM)[28] defined here as OTUs that account

for 0.1% of the total observation (sequence) count of an OTU among the 36 treatments (260,175 sequences), representing 56.6% of the initial total count. The ACM matrix, including bulk soil, rhizosphere, and root samples, was rarefied at 1,067 reads and was represented by 99 bacterial OTUs and 38,412 observations. Statistical analysis (ANOVA) was applied to count of bacterial taxa at multiple taxonomic levels.

Results

Nanoceria affect the microbial enzyme activities in the rhizosphere

We measured the activity of hydrolase and oxidoreductase soil enzymes known to be involved in the degradation of a range of substrates that are common constituents of organic matter.[29] Keystone enzymes were selected to represent carbon cycling, such as ß-glucosidase (polysaccharides, *e.g.* cellulose), peroxidase and phenoloxidase (aromatic carbon, *e.g.* lignin), as well as nitrogen cycling, including endoprotease (peptide, *e.g.* protein) and organic P such as acid and alkaline phosphatases (P monoesters). We also measured catalase activity related to aerobic metabolism. Table 1 summarizes the enzymatic activities of C, N, and P recycling in bulk soil and rhizosphere.

In bulk soil, the three types of CeO_2-NPs did not alter any of the enzymatic activities tested (as compared to controls), with the exception of bare CeO_2-U NPs, which significantly decreased catalase (by 7%; $P<0.05$). In the rhizosphere, all CeO_2-NPs significantly decreased the catalase activity and in a similar extent as compared to controls (by 19±8%, 17±3% and 22±2% for pristine CeO_2-R, CeO_2-U and coated CeO_2, respectively).

In the rhizosphere, small-size and aggregated pristine CeO_2-NPs both decreased ß-glucosidase and alkaline phosphatase activities with similar magnitudes (approximately 19-20%). The three nanoceria types did not affect any of the other assayed enzyme activities (*i.e.* protease, acid phosphatase, peroxidase and phenoloxidase). Some enzymatic activities, such as catalase (for all NPs) and alkaline phosphatase (for pristine CeO_2-R and CeO_2-U), were substantially decreased in the rhizosphere to levels observed in bulk soil. Coated CeO_2-NPs displayed the lowest effects on enzymatic activities, which included decreased catalase activity in the rhizosphere and the absence of any effect on the activity of the other assayed enzymes.

Both small-size CeO_2-R and coated CeO_2 exhibited catalase mimetic activity, whereas large-size bare CeO_2-U did not significantly reduce hydrogen peroxide activity (Fig. S2).

Nanoceria do not impact bacterial abundance or richness in the three compartments

Nanoceria did not significantly decrease the microbial biomass based on extractable DNA ($P>0.05$) in soil or the bacterial abundance ($P>0.05$), as revealed by the quantitative PCR data (Table S3).

We used alpha diversity to describe the total diversity of OTUs within the different treatment communities (CeO_2-R, CeO_2-U, coated CeO_2 and control) among the three compartments (bulk soil, rhizosphere and root). Alpha diversity was summarized, taking into account measures of richness based on qualitative Chao1 and quantitative Shannon estimators, as well as phylogeny-based measures such as Faith's PD. Figure S2 shows rarefaction curves for OTUs based on observed OTUs and distance box plots for Chao1, Shannon and PD estimators. The Chao1, Shannon index, and PD measures revealed that none of the CeO_2 NP treatments affected the alpha diversity of the microbiome (as compared to the controls) in the root compartment, the rhizosphere or bulk soil, when controlling for compartment status. A significant increase was observed for PD in the bulk soil microbiota of the CeO_2-U treatment as compared to CeO_2-R (Fig. 2; $P=0.014$ and $P=0.024$ in observed OTUs and PD, non-parametric two-sample t test).

Nanoceria greatly affect the root microbiota

In order to identify nanoceria treatments associated with compositional dissimilarity in microbiota, we examined the β-diversity, which is a measure of diversity between samples. Principal coordinates analysis (PCoA; Fig. 1a) and PERMANOVA analysis of unweighted UniFrac distances revealed clustering of microbial communities based on the soil compartment factor ($P=0.0001$), but not on nanoceria treatment ($P=0.32$). Taking into account the relative abundance of organisms, PCoA of weighted UniFrac (Fig. 1b) and Bray-Curtis (Fig. 1c) distances showed clustering based on compartment ($P=0.0001$ and $P=0.001$, respectively; PERMANOVA) but not on treatment ($P=0.15$ and $P=0.16$, respectively). Jackknifed PCoA analysis of unweighted and weighted UniFrac and Bray-Curtis distance metrics resulted in similar conclusions (Fig. S3).

Since the "compartment" factor appeared as a main variable causing change in the bacterial microbiota, we assessed the effect of nanoceria treatment within the compartment subgroups. Constrained permutations within compartments showed clustering of microbiota based on treatment for the weighted UniFrac distance (P=0.009) and Bray-Curtis distance (P=0.0129) but not the unweighted UniFrac distance (P=0.08).

PERMANOVA analysis of UniFrac distance matrices revealed that CeO_2-U induced significant differences in roots, when controlling for compartment status (Table S4). Taking into account compartment status, CeO_2-U was observed to induce significant changes in root bacterial microbiota, as compared to both the control (P=0.002) and CeO_2-R (P=0.005), based on weighted UniFrac distances., and significant changes in root bacterial microbiota, in comparison to the control (P=0.042) and coated CeO_2 (P=0.022), when using unweighted UniFrac distances (Table S4). Regarding the analysis of the weighted or unweighted UniFrac distances, nanoceria treatments did not affect the dominant microbiota in bulk soil or in the rhizosphere (as compared to the control; data not shown). As the rarefaction curve did not plateau, we may miss differences that exist in the rare biosphere, which is currently not quantifiable.[28]

Nanoceria significantly modulate the relative abundance of bacterial taxa in the rhizosphere

Figure 2 displays the bacterial microbiota composition by phylum in the root compartment. Statistical analysis (Kruskal-Wallis) exposed significant differences in the microbiota composition at the phylum level for the different treatments, when controlling for compartment status (Table S5). In roots, significant differences in the relative abundance of bacterial taxa clustered CeO_2-U from control treatment. The abundance of the phyla *Fibrobacteres*, *Chloroflexi* and *Proteobacteria* significantly increased in the CeO_2-U treatment (0.16%, 1.2% and 64.1%, respectively) in comparison to the control (0.02%, 0.66% and 42.8%, respectively), whereas the abundance of the *Actinobacteria* phylum decreased in the CeO_2-U treatment (6.3%), as compared to the control (37.4%).

In the root compartment, three phyla differentiated the bacterial microbiota of CeO_2-U as compared to CeO_2-R, with a decrease in *Actinobacteria* (from 21% to 6.3%, P=0.049, FDR=0.23), an increase in

Chloroflexi (0.63-1.2%, P=0.049) and *Proteobacteria* (54.6- 64.1%, P=0.049), and an increase in the FDR threshold (0.23).

The phylum *Armatimonadetes* and candidate phylum TM7 were significantly decreased in the CeO_2-R treatment as compared to the control (0.16-0.01%, P=0.046 and 0.75-0.32%, P=0.049, respectively), whereas a compensatory increase was observed in *Proteobacteria* and *Verrumicrobia* (42.7-54.6%, P=0.049 and 1.6-2.3%, P=0.049, FDR=0.17). No significant differences were found in bulk soil or in the rhizosphere compartment (P-value <0.05 although Q value >0.25, data not shown).

OTU richness in soil is the result of a large number of low-count OTUs.[28] Follow-up analysis focused on the community members that accounted for more than 0.1% of the total OTU counts in the matrix rarefied to 1,067 sequences. Certainly, conclusions based on the abundant community members do not account for the contribution of low-abundance taxa. These rare biosphere taxa have important ecological roles, as reservoirs of genetic and functional diversity, in the resilience of ecosystems.[30]

Statistical analysis (one-way ANOVA) revealed significant differences in the relative abundance of bacterial taxa at multiple taxonomic levels in the root compartment (Table S6). Among all treatments, some OTUs discriminated CeO_2-U from controls and CeO_2-R treatment.

In CeO_2-U *vs.* control, microbiota data revealed a severe decrease in the mean relative abundance of *Janthinobacterium*, *Kribbella*, *Micrococcaceae*, *Streptomyces* and *Pseudoxanthomonas*, and an increase in *Acidovorax*, *Pelomonas* and *Methylibium*. In CeO_2-U *vs.* CeO_2-R, mean relative abundances were reduced for *Janthinobacterium*, *Micrococcaceae* and *Variovorax*, whereas they were increased in *Acidovorax* and *Methylibium*.

Acidovorax and *Pelomonas* were barely detected in bulk soil (<0.001%) and the rhizosphere (0.006%)[31], although they were highly enriched in the root compartment of the CeO_2-U treatment. In CeO_2-U treatment *vs.* control, the OTUs affiliated to *Acidovorax* accounted for 5.65% (vs. 0.22%), *Pelomonas* for 7.65 % (vs. 1,69%) and *Methylibium* for 38\33% (vs 7.6%). *Methylibium* was the most abundant genus that could discriminate CeO_2-U treatment from the control and CeO_2-R, with an 80-fold enrichment from the bulk soil compartment to the root compartment.

Addition of nanoceria to soil at 1 mg.kg^{-1} does not impact plant growth

Canola plants were grown from seeds for four weeks in a clay-loam calcareous soil enhanced with pristine (CeO_2-R or CeO_2-U) or citrate-coated CeO_2-NPs at a concentration of 1mg/kg (dry soil mass). Plant growth was monitored by measuring the dry biomasses of below and aboveground plant parts (Fig. S5). None of the CeO_2 NP treatments affected plant growth (one-way ANOVA; P=0.85 for shoots and P=0.55 for roots).

Discussion

Nanoceria trigger functional differences that do not correlate with changes in bacterial microbiota composition

Soil enzymatic activities are recognized sensors of natural and anthropogenic disturbances occurring in the soil ecosystem. They play a crucial role in nutrient cycling and in organic matter decomposition. Furthermore, any dysfunction in the enzymatic activity of soils may disturb the biological equilibrium of soil, which may have ecological and economic consequences.[32]

In the current study, the addition of CeO_2-NPs to soil was found to lower several hydrolytic and oxidoreductive soil microbial activities. The three different NPs reduced catalase activity in the rhizosphere, with the largest pristine CeO_2-U NPs extending this effect into bulk soil. Catalase activity may be related to the metabolic activity of aerobic organisms and has been used as an indicator of soil fertility.[33] The decrease in catalase activity resulting from the NP treatments suggests an inhibition of aerobic bacterial microbiota. The intrinsic catalase mimetic activity of CeO_2-R and coated CeO_2-NPs could minimize the decrease in the measured enzymatic activity. In the rhizosphere, as compared to controls, pristine small size CeO_2-R and large size CeO_2-U decreased the ß-glucosidase activity, which is key in the last limiting step of cellulose degradation (C cycle), and reduced the alkaline phosphatase activity, which is crucial in organic P transformation.

Based on the quantitative Shannon and qualitative Chao1 richness estimators and the divergence-based PD values did not reflect any impact from CeO_2 NP treatments on bacterial microbiota richness in the bulk soil and rhizosphere compartments, as compared to the controls. Based on UniFrac metrics, which calculate a distance measure between communities using phylogenetic information,[34] the

diversities observed in bulk soil and the rhizosphere were comparable between NP treatments and controls. Based on the quantification of 16S rRNA gene copies (Table S3), NP treatments did not alter the size of bacterial communities. Many studies have shown some impact of NPs on soil enzymatic activities, often on microbial biomass and diversity. For example, Wang et al. demonstrated that soil catalase activity was significantly decreased by nano-Fe_3O_4 as well as urease activity.[35] Fe_2O_3 and ZnO NPs can effectively maintain various soil microbiological processes at 100 $mg.kg^{-1}$, however higher concentrations (*e.g.* 500-1000 $mg.1kg^{-1}$) have negative impacts on soil ecology.[36] Accordingly, soil protease, catalase and peroxidase activities were inhibited in the presence of the TiO_2 and ZnO nanoparticles.[37] Moreover, CuO NPs added to a sandy loam (at 1 and 10 $mg.kg^{-1}$) had a strong effect on bacterial hydrolytic activity.[9] These studies were conducted on a time-scale comparable[35,36] or on much longer periods of time[9,37], however at higher doses of NPs. In our study, enzymatic activity changes observed in bulk soil and the rhizosphere treated with NPs (in the presence of a low dose of CeO_2-NPs) did not correlate with a modification in bacterial community structure or size. Rather, these changes were linked to an inhibition of their enzymatic activities (potentially in aerobic bacteria), reduction of bacterial activity (that is undetected using a DNA-based approach), or to changes in microbial communities other than bacteria.

Canola potentiates the nanoceria impact on microbial activity and community structure

Measuring the activity of several enzymes in soil is a suitable method to estimate the overall microbial activity and its response to prevalent pollution.[38] Our results indicate that catalase, β-glucosidase and alkaline phosphatase activities were inhibited by pristine nanoceria at 1 $mg.kg^{-1}$. These activities were gradually impacted in terms of magnitude and type of the enzymes affected, progressing from the bulk soil to the rhizosphere.

Similarly, we found a significant impact of pristine CeO_2-U on the selection of the microbiota in the root compartment, based on the analysis of UniFrac distance metrics, phyla, and OTUs. Nanoceria did not significantly affect the microbiota of bulk soil or the rhizosphere, in comparison to controls. Thus, based on soil enzyme activities and microbial community composition, we have uncovered a

'nanoceria impact gradient' progressing from bulk soil to the root-soil interface, indicating that the plant potentiates the impact of these NPs in soil.

Terminal restriction fragment length polymorphism (T-RFLP) and PhyloChip analyses were recently used to determine that nano-CeO_2-NPs (100 $mg.kg^{-1}$) do not affect soil bacterial communities in unplanted soils, although they do trigger shifts in soybean rhizosphere communities.[11] No OTUs were significantly correlated with nano-CeO_2 treatment, and the authors interpreted these results in terms of an indirect effect of nano-CeO_2 on plant growth. Although the study did not quantify or analyze plant root exudates, the authors suggested that the exudation could be reduced, due to the stunted growth of plants exposed to 100 $mg.kg^{-1}$ of nano-CeO_2) or modified in composition, due to an abiotic stress of CeO_2-NPs on plant roots. However, in our conditions, canola growth was not reduced based on the above and belowground biomasses. We therefore conclude that the effect of CeO_2-NPs on plant root microbiota is not only mediated by an effect on the plant, but that it could also originate from a direct effect on bacteria. Pelletier and al. showed a size-dependent effect on the growth inhibition of *Escherichia coli* by nanoceria.[39]

Nanoceria generate microbiota with double-edged potential significance to plant and environmental health

Our use of quantitative PCR and alpha diversity estimators indicates that the three nanoceria examined in this study did not alter biomass or bacterial diversity, as compared to the controls. However, pristine large-size CeO_2-U NPs significantly affected the community structure (in comparison to controls), as seen in the analysis of UniFrac distance metrics. CeO_2-U treatment resulted in an increase in *Burkholderiales*, in the *Comamonadaceae* family, especially in *Acidovorax* (5.65%), *Pelomonas*, and *Methylibium* genera. (Table S6). In the root compartment, the total abundance of these OTUs reached 68.06% in the abundant community members for CeO_2-U treatment vs 9.51% in the controls (Table S6). Alternatively to Ribosomal Database Project (RDP) classifier pipeline, OTU sequences can be resolved using NCBI's Taxonomy. Based on BLAST taxonomic assignment, the closest relatives to these OTUs were *Acidovorax radicis N35, Pelomonas saccharophila*, *Methylibium petroleiphilum PM1* and *Methylibium fulvum* (reclassified as *Rhizobacter fulvum*).[47] All these bacteria are reported as

391 degraders of polycyclic aromatic hydrocarbons (PAHs) in soil.[48-55] Triggering the rise of microbial
392 genera that potentially degrade PAHs can be advantageous for remediation of hydrocarbon in soils.
393 However, hydrocarbon-degrading properties in bacteria can come with heavy metal tolerance, as well
394 as resistance to antibiotics and multidrug resistance[40-42], potentially in *Methylibium* and *Acidovorax*
395 genera.[43, 44] A functional gene analysis is needed to demonstrate this hypothetical trend. Yergeau et al.
396 identified *Methylibium* in a microbial community active in the rhizosphere of willow planted in HAP-
397 contaminated soils.[45] They show that genes related to hydrocarbon degradation together with antibiotic
398 resistance were more expressed in the HAP-contaminated rhizosphere. This underscores the need for
399 caution when disseminating ENMs that could exert a selection pressure conducive of microbial
400 blooms potentially resistant to heavy metals and antibiotics.
401 CeO_2-U NPs significantly decreased the abundance of OTUs in *Actinobacteria* (including the
402 *Actinosynnemataceae* and *Micrococcaceae* families and *Streptomyces*), *Gammaproteobacteria* and
403 *Betaproteobacteria* within the *Burkholderiales (Janthinobacterium)*, and *Xanthomonadales*
404 (*Pseudoxanthomonas*) families (Table S6). These taxa are have been identified as the most dynamic
405 taxa associated with disease suppression in soil.[46] Among the ACM, *Janthinobacterium*
406 (*Oxalobacteraceae*) was enriched in the root compartment of control plants (12.4%), but inhibited in
407 the CeO_2-U treatments (3.0%). A significant decrease in the *Streptomyces* and *Pseudoxanthomonas*
408 genera was observed in the root compartment of the CeO_2-U treatment (0.7% and 0.6%, respectively),
409 in comparison to the control (6.1 and 4.6%, respectively). *Janthinobacterium*, *Streptomyces* and
410 *Xanthomonadaceae* are identified in consortia associated with soil suppressiveness against plant
411 pathogens.[47-50] Importantly, disease suppressiveness of soil can help to reduce pesticide use.
412 *Janthinobacterium* and *Xanthomonadaceae* are both dominant taxa that exhibit a positive association
413 with plant shoot weights in wheat,[51] peach tree[50] and rice,[52] suggesting a beneficial role for these
414 bacterial groups. Thus, CeO_2-U treatment mostly affected families or genera that are beneficial to
415 plant health and growth.
416
417 ***Nanoceria design determines their impacts on microbial enzyme activities and bacterial community***
418 ***structure***

In this study, we aimed to examine how particle size or coating could modulate the impact of NPs on microbiota activity and composition in bulk soil, rhizosphere, and root compartments.

This work revealed a gradient effect for the impact on microbial activity and community structure, according to the design of nanoceria. Constrained PERMANOVA of the unweighted UniFrac distance showed significant differences in coated CeO_2-NPs vs CeO_2-R, and in CeO_2-R vs CeO_2-U, in the rhizosphere (Table S4). These results demonstrate that particle size and coating trigger differences in the microbiomes. However, even if some phyla or OTUs were able to significantly discriminate between these treatments (P-value <0.05), the Q-values surpassed the FDR 0.25 threshold (data not shown).

Pristine aggregates of CeO_2-U NPs significantly altered the microbiota composition in the root compartment in comparison to controls, CeO_2-R and coated CeO_2, whereas pristine small CeO_2-R NPs did not show any impact (in comparison to the controls). Thus, particle size appears to be a key parameter in modulating the fate of CeO_2-NPs in the rhizosphere, as well as access to the soil-root interface. This conclusion needs to be confirmed with other NPs before being extended.

The mobility of CeO_2-NPs in soil depends on the soil organic matter content, and on the surface coating of the NPs. Recent work has shown that alginate-coated NPs have higher mobility than uncoated NPs, in sandy loam soil with very low organic matter content.[53] However, surface coating decreases the NP mobility in soil solution, when the soil is enriched in organic matter. Exudation of small organic molecules increases the concentration of organic ligands in the vicinity of the root system. The competition of these organic molecules for adsorption onto the cerium surface is certainly a factor that will increase the mobility of uncoated nanomaterials.

Water moves through the soil to the plant root and then to the transpiring leaves along pressure gradients: these comprise suction (negative pressure) gradients in the soil, and diffusion pressure deficit gradients in the plant.[54] It can therefore be assumed that NP diffusion in soil originates from these pressure gradients. One recent study of porosity and hydraulic conductivity of the rhizosphere has shown that bulk soil is better at retaining water than the rhizosphere.[55] Soils influenced by roots (rhizosphere soil) are less porous due to increased aggregation, in comparison to bulk soil. Based on size exclusion, it is likely that large-size and aggregated CeO_2-U NPs can diffuse more easily at the

447 root interface, whereas smaller size NPs could be hindered in their interaction with sites inside the
448 microporosity zone.[56]
449 As stated above, pristine aggregates of CeO_2-U enabled the selection of bacterial genera those
450 members are identified as PAH degraders. Bacteria that degrade PAHs utilize an efficient oxidase to
451 promote the first step of hydrocarbon conversion, producing reactive oxygen species. Potent
452 mechanisms for reducing oxidative stress are thus required in all aerobic microorganisms that produce
453 oxygenase-type enzymes to metabolize pollutants.[57] CeO_2-NPs (IV) display a catalase mimetic activity
454 that is resistant to phosphate anions, pH changes and composition of cell culture media.[23] As indicated
455 by XANES,[20-22] the nanoceria used in this study have a (IV) oxidation state. CeO_2-R and coated CeO_2
456 exhibited catalase mimetic activity *in vitro*, which are likely to be retained in soil. Among the
457 nanoceria that we examined, only CeO_2-U, which lacks catalase activity, promoted the selection of
458 genera comprising hydrocarbon degraders potentially endowed with resistance to oxidative stress.
459 Many previous studies have shown that the catalase activity of nano-CeO_2 eases the impact on
460 organisms.[58-61]
461 Pristine small-size CeO_2-R and aggregates of CeO_2-U, which display comparable surface charges, had
462 similar effects on the hydrolytic enzymes tested. The two pristine NPs both decreased ß-glucosidase
463 and alkaline phosphatase enzyme activities, suggesting comparable interactions between the two types
464 of pristine NPs and the exocellular enzymes investigated (independent of particle size and initial
465 aggregation state). Conversely, citrate-coated CeO_2 particles did not affect these activities.
466 Compositional differences in microbial communities were not responsible for the observed functional
467 differences of CeO_2-NPs in the rhizosphere. Charge interactions could thus explain the inhibition of
468 these hydrolytic exocellular enzymes. Indeed, CeO_2-NPs must be in direct contact with cells in order
469 to display toxicity, *via* the reduction of Ce(IV) to Ce(III).[22, 62] Citrate coating tends to decrease the
470 interaction of NPs with cells, as well as the Ce reduction kinetics.[63] However, positively charged
471 CeO_2-NPs become negatively charged in soil, due to the adsorption of phosphate ions or citrate (and
472 potentially carbonate ions, in the case of an alkaline soil).[64] These new negatively charged sites at the
473 surface can promote hetero-aggregation of CeO_2-NPs with natural colloids, such as clays or metallic
474 oxides. Citrate-stabilized and bare Ag-NPs showed similar sorption to silt particles in low-carbon soil,

475 suggesting that the surface charge does not control Ag-NP sorption to silt.[65] Nevertheless, the citrate
476 ligand is capable of forming a chelate through more than one coordinating group. Therefore, this
477 coating could act as a cross-linker to associate CeO_2-NPs with colloids in a different way than the
478 negative surface charges promoted by phosphate or carbonate ions.
479 Citrate-coated NPs tend to have a low effect on enzyme activities and on the bacterial community
480 structure. However, this lack of an impact may be misleading in the vicinity of the root, due to the
481 high variability in most of the parameters measured in the rhizosphere and the root compartment
482 (biomass in the rhizosphere, UniFrac distance metrics and relative abundance of phyla in the root
483 compartment) for citrate-coated CeO_2-NP treatment (Table S3, Fig. 1, Fig. S4), which precluded
484 significant differences with other treatments. Plant roots release small organic acids that can
485 remobilize organic compounds, such as HAPs by desorption from soil aggregates.[66] Citrate coating of
486 NPs can readily exchanges with dissolved organic matter (DOM) in soil.[67] We hypothesize that a
487 progressive and random release/exchange of the coating with DOM near the root, allows NPs to
488 interact with different colloid fractions, ions or cells, which triggers a variable behavior in the
489 rhizosphere and root compartment. We hypothesize that a progressive and random release/exchange of
490 the coating, near the root, allows NPs to interact with different colloid fractions, ions or cells, which
491 triggers a variable behavior in the rhizosphere and root compartment.
492 Altogether, our results show that nanoceria reduce microbial enzymatic activities and alter the
493 bacterial community structure, and that these effects spatially increase along a gradient progressing
494 from bulk soil to the root compartment. The design of nanoceria, including particle aggregation and
495 coating, clearly determines the amplitude of this impact. The primary effect occurred for aggregated
496 pristine nanoceria, which were deprived of catalase activity. Contamination of soil with pristine
497 aggregates of nanoceria conferred a selective advantage to genera whose common members are
498 hydrocarbon-degrading bacteria, which are potentially more resistant to oxidative stress, heavy metals
499 and antibiotics. On the other hand, several families and genera, whose membres can be involved in
500 disease-suppressive activity toward plant pathogens, were inhibited in the root compartment of canola.
501 The finding that these effects occurred at the lowest dose ever tested in soil (1 $mg.kg^{-1}$) is of particular
502 concern, and invites further research in order to identify the resilience of the ecosystem. Nevertheless,

when 4-nm sized CeO_2-NPs were tested, the impact of nanoceria was limited to enzymatic activities. This shows that nanoceria can be designed to ease the impact on soil microorganisms.

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Supporting Information

Supporting Information includes detailed protocols of enzymatic activity experiments, sequencing and post-run analyses, catalase mimetic activity of nanoceria, microbial diversity (PCoA plots and composition at the phylum level), plant biomass, physicochemical properties of the nanoceria and oil used in these experiments, and bacterial biomass. This material is available free of charge *via* the internet at http://pubs.acs.org.

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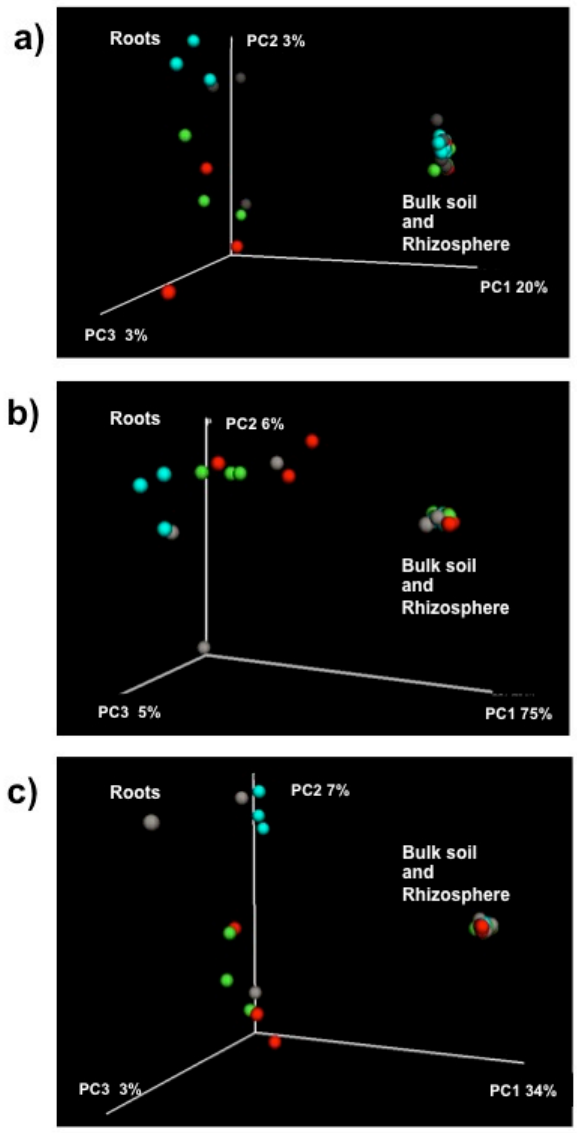

Figure 1: 3D beta diversity presented as PCoA plots depicting the clusters of bacterial communities within the different compartments (left: roots; right: rhizosphere and bulk soil) for the nanoceria treatments CeO_2-R (green), CeO_2-U (blue), coated CeO_2 (grey) and controls (red). Unweighted UniFrac (a), weighted UniFrac (b), and Bray Curtis (c) distance metrics. The percent variation explained by the PCs is indicated on the axes and refers to the fraction of the total variance. Statistical analysis (PERMANOVA) of unweighted and weighted UniFrac as well as Bray-Curtis distance metrics revealed clustering of microbial communities based on the soil compartment factor (Fig. 1a, P=0.0001; Fig. 1b P=0.0001; Fig. 1c, P=0.001) but not on nanoceria treatment (P>0.05).

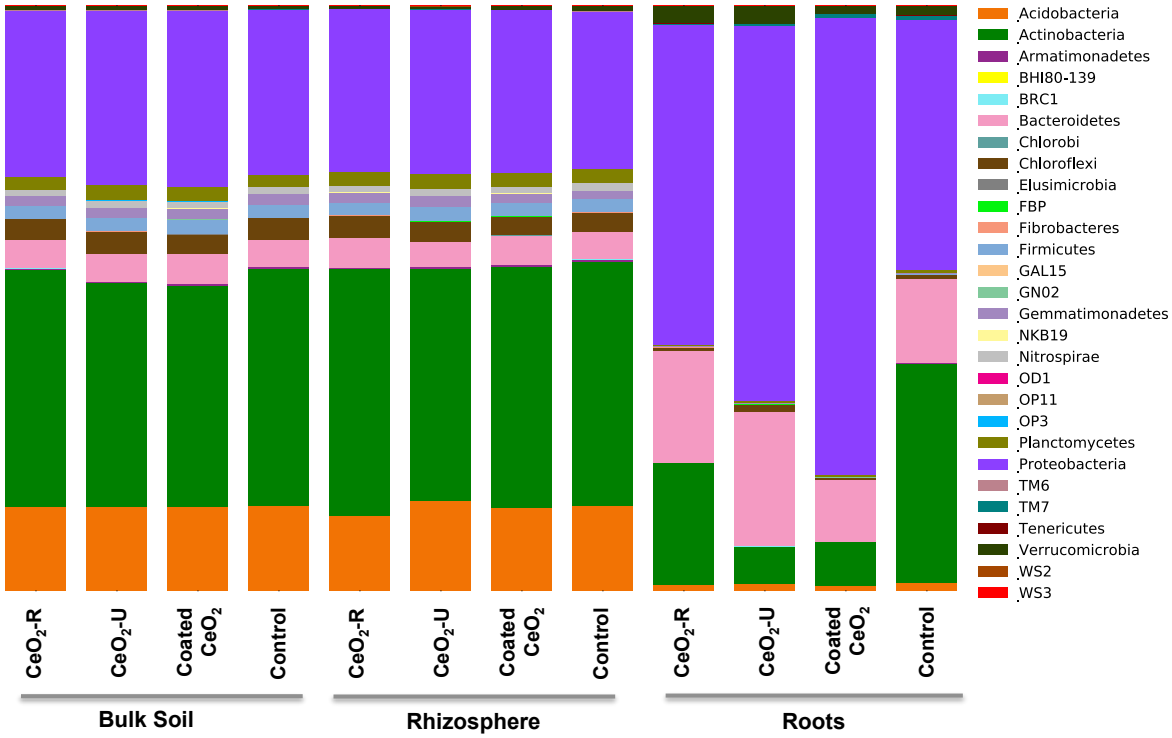

Figure 2: Relative abundance of major phyla in bulk soil, rhizosphere and root compartments for soil enhanced with nanoceria (pristine CeO_2-R, CeO_2-U and citrate-coated CeO_2) at 1 $mg.kg^{-1}$ and the respective controls. Significant differences were found in the root compartment and are summarized in Table S5.

Table 1: Enzymatic activities in the rhizosphere of canola and in bulk soil after 30 days of exposure to nanoceria. Samples include pristine CeO_2-R and CeO_2-U, and coated CeO_2 (1 $mg.kg^{-1}$) and controls. Values in bold denote statistically significant differences from controls (one-way ANOVA, post-hoc Student-Newman-Keuls test; $P<0.05$)

Enzymatic activity	Rhizosphere				Bulk Soil			
	Control	CeO_2-R	Coated-CeO_2	CeO_2-U	Control	CeO_2-R	Coated-CeO_2	CeO_2-U
Catalase								
µmol $KMnO_4$ g^{-1} dry soil min^{-1}	5.4 ± 0.4	**4.3 ± 0.6**	**4.2 ± 0.2**	**4.4 ± 0.1**	3.9 ± 0.3	3.7 ± 0.2	3.5 ± 0.3	**3.2 ± 0.1**
β–Glucosidase								
µmol p-NP g^{-1} dry soil h^{-1}	1.28 ± 0.01	**1.037± 0.07**	1.16 ± 0.08	**1.05 ± 0.11**	0.93 ± 0.07	0.86 ± 0.03	1.06 ± 0.01	0.90 ± 0.05
Peroxidase								
µmol h^{-1} g^{-1}	0.170 ± 0.002	0.154 ± 0.014	0.152 ± 0.006	0.168 ± 0.015	0.158 ±0.030	0.129 ± 0.023	0.157 ± 0.033	0.145 ± 0.003
Phenoloxidase								
µmol h^{-1} g^{-1}	0.221 ± 0.008	0.213 ± 0.03	0.210 ± 0.004	0.214 ± 0.014	0.238 ± 0.029	0.189 ± 0.058	0.194 ± 0.038	0.173 ± 0.004
Acid Phosphatase								
µmol p-NP g^{-1} dry soil. h^{-1}	0.78 ± 0.03	0.71 ± 0.06	0.72 ± 0.08	0.69 ± 0.11	0.63 ± 0.04	0.60 ± 0.08	0.72 ± 0.15	0.59 ± 0.01
Alkaline Phosphatase								
µmol p-NP g^{-1} dry soil. h^{-1}	0.90 ± 0.04	**0.73 ± 0.10**	0.79 ± 0.05	**0.73 ± 0.04**	0.63 ± 0.03	0.61 ± 0.02	0.65 ± 0.03	0.61 ± 0.07
Protease								
µg l-tyrosine released . g^{-1} dry soil h^{-1}	133 ± 33	105 ± 16	119 ± 18	123 ± 17	93 ± 20	110 ± 16	104 ± 18	90 ± 28

p-NP: para-nitrophenyl

TOC Abstract :