**Soil organic matter influences cerium translocation and physiological processes in kidney
bean plants exposed to cerium oxide nanoparticles**

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Abstract

Soil organic matter plays a major role in determining the fate of the engineered nanomaterials (ENMs) in the soil matrix and effects on the residing plants. In this study, kidney bean plants were grown in soils varying in organic matter content and amended with 0-500 mg/kg cerium oxide nanoparticles (nano-CeO₂) under greenhouse condition. After 52 days of exposure, cerium accumulation in tissues, plant growth and physiological parameters including photosynthetic pigments (chlorophylls and carotenoids), net photosynthesis rate, transpiration rate, and stomatal conductance were recorded. Additionally, catalase and ascorbate peroxidase activities were measured to evaluate oxidative stress in the tissues. The translocation factor of Ce in the nano-CeO₂ exposed plants grown in organic matter enriched soil (OMES) was twice as the plants grown in low organic matter soil (LOMS). Although the leaf cover area increased by 65-111% with increasing nano-CeO₂ concentration in LOMS, the effect on the physiological processes were inconsequential. In OMES leaves, exposure to 62.5-250 mg/kg nano-CeO₂ led to an enhancement in the transpiration rate and stomatal conductance, but to a simultaneous decrease in carotenoid contents by 25-28%. Chlorophyll *a* in the OMES leaves also decreased by 27 and 18% on exposure to 125 and 250 mg/kg nano-CeO₂. In addition, catalase activity increased in LOMS stems, and ascorbate peroxidase increased in OMES leaves of nano-CeO₂ exposed plants, with respect to control. Thus, this study provides clear evidence that the properties of the complex soil matrix play decisive roles in determining the fate, bioavailability, and biological transport of ENMs in the environment.

Keywords: Soil organic matter, cerium oxide nanoparticles, kidney bean, gas exchange, photosynthetic pigments, phytotoxicity

1. Introduction

Along with the vast array of applications that the various classes of engineered nanomaterials (ENMs) offer, they are associated with unknown risks throughout their life cycle in different environmental conditions. The fate of ENMs in the environment depends on their physicochemical properties (like composition, particle size, surface charge, and dissolution) as well as the nature of the target environmental matrices (Cornelis et al., 2014; Peijnenburg et al., 2015). In addition, modulating the ENM properties for various biological applications such as medicine (Nguyen and Zhao, 2015) and agriculture (Kah et al., 2013), also makes them bioavailable to other non-targeted species, as they likely transport and distribute across different media (Liu and Cohen, 2014). This poses serious challenges towards a precise hazard and risk assessment of ENMs to aid regulatory measures and decision-making.

Recent computational modelling and simulation studies on the environmental fate of ENMs involving mass flow and multimedia apportionment have estimated that the major fraction of the released ENMs during production, usage, and disposal, finally accumulate in soils or sediments (Collin et al., 2014; Liu and Cohen, 2014; Keller and Lazareva 2014). This raises concerns due to the possibility of transport of the ENMs from the soils to agricultural crops and, eventually, bioaccumulation in the food chain (Judy et al., 2011; Gardea-Torresdey et al., 2014; Hawthorne et al., 2014; Majumdar et al., 2015), or leaching to the groundwater (Quik et al., 2010). Soil properties including texture, porosity, pH, ionic strength, organic matter, and mineral composition play a pivotal role in determining the aggregation, dissolution, chemical transformation, bioavailability, and migration of ENMs in the porous media, as well as in their interactions at the nano-bio interface (Schwabe et al., 2013; Cornelis et al., 2014; Garner and Keller, 2014; Read et al., 2015; Conway and Keller, 2016). Soil organic matter (SOM) is

primarily (60-80%) composed of humic acids, fulvic acids, and humin, which are direct products of plant residue decomposition (Brady and Weil, 2008). Evidences show that organic acids like humic and fulvic acids stabilize several metal oxide nanoparticles in aqueous solutions, including nano-CeO₂ (Yang et al., 2009; Schwabe et al., 2013), zinc oxide (nano-ZnO) (Zhang et al., 2009), and titanium oxide (nano-TiO₂) (Zhang et al., 2009; Domingos et al., 2009), owing to enhanced charge and/or steric stabilization (Quik et al., 2010). This prevents aggregation of ENMs, possibly leading to their stability and prolonged suspension in the soil pore water or increased mobility through plant roots. Johnson et al. (2009) demonstrated enhanced mobility of nano-zero valent iron (nZVI) in granular media in the presence of natural organic matter.

Due to increasing potential applications of nano-CeO₂ in electronics, catalysis, fuel additives, agriculture, and medicine (Collin et al., 2014), recent phytotoxicity studies are more focused on realistic exposure conditions (Holden et al., 2016). Consolidation of several soil-based studies with nano-CeO₂ may reach to a consensus that although, it is actively transported within plants, it is less detrimental in terms of effects on growth and metabolic processes compared to other metal oxide nanoparticles (Wang et al., 2012; Priester et al., 2012; Zhao et al. 2013, 2015). However, negative effects have been also reported on nitrogen fixation in soybeans (Priester et al., 2012), crop yield in corn, cucumber and soybeans (Zhao et al. 2013, 2015; Priester et al., 2012), and also on the nutritional quality of the edible seeds in different plants like rice (Rico et al., 2013) and kidney beans (Majumdar et al., 2015). Surprisingly, filial generations of *Brassica rapa* plants were shown to experience higher oxidative stress compared to parent plants (Ma et al., 2016). Although the literature on the fate and toxicity of nano-CeO₂ keeps on growing exponentially, there have been very few investigations directly addressing the role of SOM in the nano-plant interactions under natural conditions. Schwabe et al. (2013) reported that

supplemented organic matter enhanced root adsorption of Ce in pumpkin and wheat plants exposed to 100 mg/L nano-CeO₂ suspended in Hoagland culture medium. In corn plants, lower Ce translocation to aerial tissues was observed in organic matter rich soil, compared to natural unenriched soil amended with bare as well as alginate-coated nano-CeO₂ (Zhao et al., 2012). According to Zhang et al. (2016), silty loam soil with lower SOM content (~2%) had more exchangeable/bioavailable fraction of Ce, leading to higher translocation to radish shoots, compared to loamy sand soil (~12%). This suggests that soil components have a high potential to modulate the surface properties of ENMs. This modulation can change the transport within plants and the effects of ENMs in plant health and metabolic processes. Extensive research is needed in order to fully understand these phenomena.

To the best of the authors' knowledge, there are no existing reports investigating SOM dependent toxicological response in plants exposed to nano-CeO₂. In this study, we examined the effect of nano-CeO₂ at varying concentrations on physicochemical properties of the soils and the effects on kidney bean plants grown for 52 days from germination. The Ce uptake by roots and further translocation to aerial parts were analyzed using inductively coupled plasma-optical emission spectroscopy. We examined varying SOM content as a factor of the changes in various physiological markers like net photosynthesis, stomatal conductance, transpiration, and leaf area. Additionally, photosynthetic pigments and the activities of antioxidant enzymes in different tissues were assessed to investigate stress response in the plants.

2. Materials and methods

2.1. Cerium oxide nanomaterials

The nano-CeO₂ particles (~8 nm, Meliorum Technologies, Rochester, NY) were procured from The University of California Center for Environmental Implications of Nanotechnology (UC-CEIN). Keller et al. (2010) characterized these nanoparticles as 100% cubic ceria nanorods (95.14% pure), measuring $(67 \pm 8) \text{ nm} \times (8 \pm 1) \text{ nm}$, ($\leq 10\%$ polyhedral; $8 \pm 1 \text{ nm}$) with a surface area of $93.8 \text{ m}^2 \text{ g}^{-1}$. Suspensions of nano-CeO₂ in Millipore water (MPW) were prepared using 30 min bath sonication and characterized for size and zeta potential using Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, U.K.). The measurements were done in triplicates, each with three consecutive readings for technical replication.

2.2. Soil collection and preparation

Natural soil was collected from an agricultural field in Fabens, TX (N 31° 29' 02.1", W 106° 08' 27.2", elevation 1102 meters, mineral horizon, depth 55 cm). The soil was classified as sandy loam soil (64% sand, 31% silt, 5% clay) according to USDA soil texture classification. Half portion of the collected soil was amended with Miracle-Gro potting mix at 2:1 (v/v) ratio to enhance the SOM content. Based on comparative soil organic matter (SOM) content (Majumdar et al., 2015), the collected natural soil (4.2%) was called low organic matter soil (LOMS) and the potting mix added soil (10.1%) was called organic matter enriched soil (OMES). According to Majumdar et al. (2015), soil characteristics including pH, electrical conductivity (EC), total dissolved solid (TDS), and cation exchange capacity (CEC) of LOMS were 7.9 ± 0.1 , $208 \pm 4.8 \mu\text{S/cm}$, $23.3 \pm 1.8 \text{ meq/100g}$ and $92.5 \pm 2.5 \text{ mg/L}$, respectively; and of OMES were 7.3 ± 0.1 , $485 \pm 35.7 \mu\text{S/cm}$, $33.1 \pm 1.3 \text{ meq/100g}$ and $240 \pm 19.2 \text{ mg/L}$. Soil bulk density was measured following McKenzie et al. (2004). Water holding capacity (%) of the soils were also calculated to estimate the total volume of the applied nanosuspensions, as follows: $[\{\text{Mass of water to saturate the soil (g)}/\text{Oven dry weight of the soil (g)}\} \times 100]$. The water holding capacity for

LOMS and OMES was 17 and 23 %, respectively. Addition of potting mix as a source of SOM however also resulted in enrichment of some nutrients like N, S and Fe.

2.3. Exposure scenario of nano-CeO₂ to kidney bean plants

As previously described (Majumdar et al., 2015), suspensions of nano-CeO₂ in MPW were prepared upon 30 min bath sonication at 25 °C (120 V, 3 Amps, 50/60 Hz). The total volume of the suspensions was adjusted to slightly smaller volume than the water holding capacity of the respective soils to prevent leaching and mass loss of the particles in each pot volume. The suspensions were mixed manually with the soils to achieve homogeneity and final concentrations of 62.5, 125, 250, and 500 mg/kg in 4 and 3 kg of LOMS and OMES, respectively. The treated soils were then placed in 7 1/2" dia. x 6" H plastic pots in quadruplicates, and were allowed to equilibrate for 24 h. Soils mixed with only MPW were used for untreated controls.

Kidney bean (*Phaseolus vulgaris* var. Red Hawk) seeds provided by Dr. James Kelly from Michigan State University, were washed with 2% sodium hypochlorite and MPW. Prior to sowing, the seeds were pre-soaked for 24 h in MPW. Fifteen seeds were equidistantly placed in each pot at a depth of 2.5 cm from the surface. Initially, the pots were placed in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14 h photoperiod (340 $\mu\text{mole m}^{-2}\text{s}^{-1}$), 25/20°C day/night temperature and 65-70% relative humidity for closer observation for 7 days until the appearance of the cotyledonous leaves. The pots were then transferred to a greenhouse with similar condition for 45 days. The plants in each pot were watered every day with 200 mL MPW for 15 days after planting. After the appearance of the first true leaves, the volume of irrigation water was increased to 400 ml until harvest, with no significant leaching in any treatments.

2.4. Soil characterization after nano-CeO₂ treatment

Five grams of control and treated soils collected before sowing (BS) kidney bean seeds were air-dried. The BS soils were homogenized and sieved through 2 mm mesh prior to characterization. Although on sieving, nothing was retained in the 2 mm strain for LOMS, large aggregates of SOM was separated from OMES, which have been referred as OMES-OM in the following sections. All the fractions were stored for elemental analysis. Soil pH in water and CaCl₂, EC and TDS were measured using Hanna Instruments HI 9813-6 Portable pH/EC/TDS/Temperature Meter. Cation exchange capacity (CEC) was measured by EPA Method 9081 (USEPA, 1986). Nitrogen content in the soils were determined using Kjeldahl method using a digestion and distillation unit (Labconco Co., Kansas city) and expressed as % N (NTK) (Bremner, 1996). Soil micro- and macro-nutrient and Ce contents were also determined as discussed in section 2.7.

After 52 days of plantation, a 12 cm deep soil column was sampled from each pot using a soil core sampler (14 cm x 1.5 cm). The sampler is shown in the Supplementary Material (SM) Illustration S1. Each soil core was divided into three parts depending on the depth from the surface, the surface zone (SZ; 0-1 cm), the rhizospheric zone (RZ; 1-8 cm), and the bottom zone (BZ; 8-12 cm), as shown in SM Illustration S1. Soil samples from the three zones, the BS soils, and sieved SOM from OMES (OMES-OM) were further dried in an oven at 60 °C for 96 h to quantify the Ce content, as discussed later.

2.5. Monitoring of plant gas exchange activities and photosynthetic pigment

Leaf net photosynthetic rate (P_n), stomatal conductance (g_s), and transpiration (E) of one plant per replicate were monitored after 52 days of exposure (DE) to 0-500 mg/kg nano-CeO₂, by placing a fully expanded leaf in the cuvette of a portable gas exchange system (CIRAS-2; PP

Systems, Amesbury, MA). The environmental conditions in the cuvette were controlled at a leaf temperature of 25 °C, photosynthetic photon flux (PPF) of 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and CO₂ concentration of 375 $\mu\text{mol}\cdot\text{mol}^{-1}$. The plants were well watered to avoid water stress, and the P_n, and g_s, E of four plants were recorded between 10:00 AM and 2:00 PM.

The major photosynthetic pigments in the leaves were extracted in 80 % acetone. Chlorophyll *a*, chlorophyll *b*, and total carotenoids were measured as described by Lichtenthaler and Buschmann (2001).

2.6. Plant harvest and growth related parameters

After 52 DE, the plants were harvested and the lengths of primary root and stem from four plants in each replicate treatments were measured. The number of leaves in ten plants per replicate was noted. The area of the first true leaf of three plants per pot was determined using LI-3100C area meter (LI-COR® Biosciences, Lincoln, NE) after 52 DE.

Two plants from each pot composited as one sample from each replicate were used to determine Ce and micronutrient contents in the different tissues of the plant. The roots were carefully washed three times alternately with 0.1% plasma pure HNO₃ and MPW to facilitate removal of adsorbed particles on the root surface and root hairs. The whole plant was then severed into roots, stems, and true leaves, which were dried for 96 h at 70 °C for further elemental analysis. The plants used for biochemical analyses were only washed thrice with MPW.

2.7. Quantification of cerium and mineral nutrients in soil and plant tissues

Dried roots, stems and leaves were digested in a mixture of plasma pure HNO₃ and 30% (w/v) H₂O₂ (1:4) using microwave accelerated reaction system (CEM Mars_x, Mathews, NC) following the U.S. EPA 3051 method (Packer et al., 2007). Accumulation of Ce and

micronutrients (Al, B, Cu, Mn, Fe, Zn) were analyzed in the digested tissues using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Optima 4300 DV, Perkin Elmer, Waltham, MA). The Ce and elemental (Al, Cu, Fe, Mn, Ni, Zn, Ca, K, Mg, P, S) contents in the aqua-regia digested soil samples (control and treated BS soils, core samples BZ, RZ and SZ of control and treated soils) were also determined using ICP-OES. Blanks, spikes, and certified standard reference material for plants (peach leaves NIST-SRM 1547) and soils (NIST-SRM 2709a San Joaquin Soil) were analyzed to validate the digestion process and spectroscopic analysis, obtaining 98% recovery for Ce. The detection limit of ICP-OES was measured to be 1 $\mu\text{g/L}$. Also, 0.5 mg/L multi-elemental standard was analyzed every 10 samples to monitor the matrix effect on the analytes and for quality assurance/quality control (Majumdar et al., 2014).

2.8. Antioxidant enzyme activity assays

The fresh plants rinsed with MPW were severed into roots, stems, and leaves. The tissues were frozen immediately in liquid nitrogen and ground to fine powder. The powdered tissues were extracted in 50 mM K-phosphate buffer containing 1 mM EDTA, 1 % PVP, and 0.5% (v/v) Triton X-100. The 10% homogenates were centrifuged at $10,000 \times g$ for 20 min (Eppendorf 5417R centrifuge, Hamburg, DE) (Gallego et al., 1996), and the supernatants were assayed to perform specific activity of catalase (CAT) and ascorbate peroxidase (APOX) according to Aebi (1974) and (Nakano and Asada, 1981), respectively. The enzyme activities were expressed as $\mu\text{mol H}_2\text{O}_2$ decomposed $\text{mg}^{-1}(\text{tissue f wt}) \text{ min}^{-1}$.

2.9. Statistical analysis

For comparison of untreated LOMS and OMES characteristics and quality, a two-tail Student t-test was used in paired comparisons. For all other grouped comparisons, the statistical analysis of the data was performed using one-way ANOVA followed by Tukey's multiple

comparisons test based on a probability of $p \leq 0.05$ (Systat SigmaPlot 13.0 software). All analyses were performed in quadruplicates. A factorial two-way ANOVA was performed to examine the interaction between the two factors: nano-CeO₂ (5 treatments: 0, 62.5, 125, 250 and 500 mg/kg soil) and soil type (2 types: LOMS and OMES) on the soil characteristics and plant physiological/biochemical parameters. Post hoc comparison was carried out by Tukey's test to establish significance difference of $p \leq 0.05$ among means.

3. Results and discussion

3.1. Effect of nano-CeO₂ on soil properties and Ce distribution in soil horizons

In this study, the amendment of the low and high organic matter containing soils with nano-CeO₂ suspensions affected their physico-chemical properties. The general properties and micro- and macro-nutrient contents of the control and nano-CeO₂ treated LOMS and OMES (sieved and OMES-OM) are shown in Table 1 and SM Table S1. The Ce content in the soils exposed to 0-500 mg/kg nano-CeO₂ is presented in Figure 1. As Ce naturally occurs as a rare earth element, 38.4 ± 0.8 and 33.8 ± 1.0 mg/kg Ce was found in sieved untreated LOMS and OMES, respectively. However, the exposure concentrations of nano-CeO₂ were at significantly higher levels. LOMS and OMES exposed to 62.5 to 500 mg/kg nano-CeO₂ treatments were analyzed for Ce content, which yielded 79.7 ± 0.6 to 386 ± 9.6 mg/kg and 74.5 ± 0.6 to 350 ± 27 mg/kg Ce, respectively in the sieved fraction (≤ 2 mm) in a dose-dependent manner (Figure 1), as expected. OMES-OM fraction accumulated 4.9 ± 2.9 to 118 ± 10 mg/kg Ce at 62.5 to 500 mg/kg nano-CeO₂ exposure treatments. In a recent study by Zhang et al. (2016), higher concentration of oxidizable fraction of CeO₂ was found in soil with higher organic matter, suggesting strong association of nano-CeO₂ in soil with SOM. As Miracle Gro potting mixes are commercially sold as mix of nutrient supplements for plants, untreated OMES (10.1% SOM)

also had significantly higher levels of macronutrients like N and S, and micronutrients like Fe, than LOMS (4.2% SOM) (Table 1). As seen in Table 1, OMES also had significantly higher EC, TDS and CEC than LOMS ($p \leq 0.05$). Thus, the OMES was more fertile and amenable for plant growth, owing to availability of higher concentrations of mineral nutrients. The addition of nano-CeO₂ to the soils however did not affect the soil pH and CEC. But, it significantly increased the EC and TDS at all the treatment concentrations in both soils, compared to their respective controls ($p \leq 0.05$) (Table 1). At 500 mg/kg, the EC and TDS increased by 127 and 149 %, respectively in LOMS and by 103 and 107%, respectively in OMES with respect to their controls. Two-way ANOVA also suggested that the effect of nano-CeO₂ treatment on the EC, TDS and % TKN in the soils depended on the SOM content *in situ*, with interaction at $p \leq 0.001$. Nanoscale materials have been used for synthesis of ceramics, metals, alloys, or semiconductors, and composite materials for various applications due to its ability to increase EC (Rittner and Abraham, 1998). Thus, an increase in EC and TDS in the soils on nano-CeO₂ addition was noted, irrespective of soil type. The measurement of both EC and TDS in the soils gives an indirect measurement of the salt content (Brady and Weil, 2008). An increase in EC and TDS signify an increase poor water filtration and drainage in the soil salinity, which may alter the soil fertility. Increased EC leads to poor water filtration and drainage thereby affecting plant health. In addition, Table 1 shows that at 250 and 500 mg/kg nano-CeO₂ exposures, Ca, Mg, Cu, Fe and Zn contents in the LOMS, and K, Mg, Al, Fe, Mn, Zn in OMES decreased significantly compared to respective untreated soils. In contrary to our observations, Conway and Keller (2016) showed that at 100 mg/kg nano-CeO₂, nano-Cu(OH)₂ and nano-TiO₂, Mg ion release was increased. However, as seen in SM Table S1, the micronutrients Cu, Fe, Mn contents were significantly higher in OMES-OM fraction of the OMES treated with 62.5 mg/kg by 59, 58 and 51%

compared to control. Sulfur content in OMES-OM was significantly higher at 125 and 500 mg/kg OMES. The enhanced micronutrient in the organic fraction of the OMES with simultaneous accumulation of Ce per se, suggests that nano-CeO₂ at low concentration may release elements more efficiently in the presence of organic matter. However, nano-CeO₂ application within each soil type did not alter the CEC (Table 1), which measures the capacity of the soil to exchange cations like Al³⁺, Ca²⁺, Mg²⁺, K⁺, NH⁴⁺, Na⁺ (Brady and Weil, 2008).

A soil core was obtained from each treatment replicate after 52 days of plant growth, which was further divided into surface, rhizosphere, and bottom zones, based on depth horizons. The Ce contents in each zone are shown in Figure 2. The ratios of Ce concentration in each zone to Ce concentration in the respective soils measured before sowing were similar (0.80 ± 0.02) between zones, soil types, and nano-CeO₂ treatments. In support of our findings, Conway and Keller (2016) also showed that nano-CeO₂ is primarily retained in the area of contamination with low rate of vertical transport in a soil column. The CEC in OMES was 43% higher than in LOMS, which suggests greater number of sites for exchange of the cations. The humus colloids in soils encourages aggregate stability and soil mineral decomposition, leading to increased availability of cationic nutrients in easily exchangeable form for the plants, without percolating through soil pores (Brady and Weil, 2008). In presence of SOM, low molecular weight organic acids such as humic and fulvic acids tend to form a coat around the nanoparticles (NPs), and stabilize them in the soil solution by electrostatic repulsion. This refrains the nanoparticles to aggregate among themselves or to soil components; thus, increasing their mobility in soil solution (Yang et al., 2009; Lin et al., 2010; Cornelis et al., 2014). However, adsorption and stabilization processes depend on the surface charge of the NPs and soil pH. In this experiment, the alkaline soil environment favors the adsorption of the negatively charged groups of SOM to

positively charged nano-CeO₂ (11 ± 2.5 mV, suspended in MPW), thereby stabilizing these particles in the pore water. In LOMS, the surface charge of the heterogeneous soil colloids allows the nanoparticles to aggregate and thereby restrict their ability to pass through the soil pores (Schwabe et al., 2013). As shown by Zhang et al (2016), the major proportion of the Ce from nano-CeO₂ in soil gets associated with the silicate minerals in the residual fraction. Moreover, at 100 mg/kg nano-CeO₂, the residual fraction holds higher concentration of Ce in soils with lower SOM (Zhang et al., 2016), which decreases with increase in exposure concentration. Significant leaching was not observed in either soil, since the irrigation water was adjusted to the soil pore volume. Instead, the Ce concentration was homogeneous along 12 cm deep soil (Figure 2). This confirms that, regardless of one time nano-CeO₂ supplementation and prolonged exposure condition, the plant roots were exposed to a homogenous Ce concentration in the soil throughout their life cycle. On closer observation, it was interesting to note that in OMES, at the highest nano-CeO₂ treatment (500 mg/kg), a progressive increase was noted in Ce content with increasing soil depth in OMES, unlike in LOMS (Figure 2). The SZ, RZ and BZ of OMES contained 362 ± 1.8 , 405 ± 30 , 424 ± 26 mg/kg Ce, respectively, whereas, in LOMS accumulated 379 ± 2.1 , 362 ± 4.2 , 359 ± 40 mg/kg Ce, respectively. However, the variation among the zones were not significant enough to exclude the possibility that the difference was due to random sampling variability. Nevertheless, this trend corroborates the possibility of increased mobility in presence of higher SOM content.

3.2. Uptake and accumulation of cerium and nutrients by the roots

The accumulation of Ce in roots of kidney bean plants grown in 0-500 mg/kg nano-CeO₂ amended LOMS and OMES for 52 days, is shown in Figure 3A. Micronutrient concentrations in roots exposed to 0-500 mg/kg nano-CeO₂ amended LOMS and OMES are given in SM Figure

S1. The Ce accumulation in roots exposed to nano-CeO₂ was dose-dependent. At 62.5, 125, 250 and 500 mg/kg nano-CeO₂, the LOMS roots accumulated 4.9 ± 0.9 , 12.2 ± 3.3 , 18.8 ± 2.8 , and 24.9 ± 1.4 mg Ce/ kg tissue d wt, respectively; and the OMES roots accumulated 2.8 ± 0.8 , 8.5 ± 2.9 , 9.7 ± 0.9 and 22.8 ± 6.8 mg Ce/ kg tissue d wt, respectively (Figure 3A). In LOMS, the root Ce concentrations were significantly higher in the plants exposed to 125-500 mg/kg with respect to control. Whereas in OMES, although the increase in root Ce concentration was dose-dependent, the accumulation was significant only at 500 mg/kg nano-CeO₂. The dose-dependent accumulation of Ce in roots was not dependent on the soil type, as deduced from two-way ANOVA test. In addition, the supplementation of nano-CeO₂ to LOMS or OMES did not affect the micro- or macro nutrient mobilization to plant roots (SM Figure S1).

The Ce accumulation in the kidney bean roots at 500 mg/kg nano-CeO₂ (24.9 ± 1.4 and 22.8 ± 6.8 mg Ce/kg d wt tissues in LOMS and OMES, respectively), were significantly lower than that reported in other soil based studies in soybeans (175 mg/kg d wt) (Priester et al., 2012), cucumber (317 mg/kg d wt) (Zhao et al., 2013), and corn (110 mg/kg dwt) (Zhao et al., 2015) at similar exposure concentrations. This could be a result of the interaction of the NPs with soil properties or species-dependent variation in response to nano-CeO₂. However, in another study where Wang et al. (2012) supplied 10 mg/L nano-CeO₂ chronically to the soil two times every week for 70 days leading to total input of 130 mg nano-CeO₂ /kg soil, the tomato roots accumulated up to 12 mg/kg d wt. The accumulation in the tomato roots corroborate to our observations at a similar exposure concentration of 125 mg/kg nano-CeO₂ in LOMS as well as OMES. High resolution imaging techniques using transmission electron microscopy and synchrotron X-ray micro-spectroscopy have provided evidences that a large fraction of nano-CeO₂ is adsorbed by the root hairs (Majumdar et al., 2014), and is internalized with minimum

biotransformation (Zhang et al., 2012; Hernandez-Viezcas et al., 2013; Majumdar et al., 2014). In LOMS, the soil colloids and minerals tend to bind to the nano-CeO₂, leading to aggregation and larger size (Quik et al., 2010; Cornelis et al., 2014), thereby reducing the mobility in the pore spaces. Thus, the nano-CeO₂ may primarily remain either incorporated in the root epidermal layers (Zhang et al., 2012; Majumdar et al., 2014) or bound to root exudates, leading to higher adsorption or localized accumulation in the roots. On the other hand, in OMES, the organic matter stabilizes the nano-CeO₂ in the pore spaces and they remain dispersed maintaining their nanoparticulate size or remaining as small aggregates (Yang et al., 2009; Schwabe et al., 2013). This enables the nano-CeO₂ to penetrate root barriers, and more accessible to the vascular tissues (Figure 3B). In contrast to our findings, Zhao et al. (2012) reported significantly lower root accumulation of Ce in the unenriched soil, compared to enriched soil. Also, radish fine roots exposed to 500 mg/kg nano-CeO₂ in silty loam soil with ~2% SOM, accumulated higher concentration of Ce, compared to loamy sand soil containing ~12% SOM (Zhang et al., 2016).

3.3. Translocation of cerium from roots to leaves

The concentration of Ce in the leaves of plants grown in LOMS and OMES amended with 0-500 mg/kg nano-CeO₂ has been provided in Figure 3B. The Ce concentration in the stems were below the detection limit of ICP-OES. Leaves in control plants grown in LOMS and OMES showed 0.13 and 0.16 mg/kg of Ce, respectively, which could be due to the presence of Ce in the natural soil. Even though at 62.5 mg/kg nano-CeO₂, Ce was not detected in leaf tissues, with increasing nano-CeO₂ exposure concentration (125-500 mg/kg), the leaves accumulated 0.19 ± 0.12 , 0.3 ± 0.1 , 0.6 ± 0.1 mg Ce/kg d wt in LOMS plants and 0.3 ± 0.1 , 0.4 ± 0.2 , 1.1 ± 0.3 mg Ce/kg d wt in OMES plants. The plants in both soils accumulated the highest Ce concentration in leaves at 500 mg/kg at a significantly higher level ($p \leq 0.05$), compared to respective controls.

Unlike in roots, the Ce content in the leaves of nano-CeO₂ exposed kidney bean plants were considerably higher than in soybean (0.000247 mg/kg d wt) (Priester et al., 2012), whereas similar to cucumber (1.72 mg/kg d wt) (Zhao et al., 2013), corn (1.84 mg/kg d wt) (Zhao et al., 2015) and tomato (0.97 mg/kg d wt) (Barrios et al., 2015) plants at 400-500 mg/kg nano-CeO₂. As shown in SM Table S2, Ce translocation factor in kidney bean plants was 0.02-0.03 in LOMS and 0.04-0.05 in OMES. Furthermore, the translocation of Ce to the kidney bean leaves was 65, 44, 71% higher in OMES than LOMS at 125, 250 and 500 mg/kg nano-CeO₂ treatments. This indicates that the Ce translocation to the aerial tissues in the kidney bean plants was enhanced by the increase in SOM. Dissolved organic matter in soil has been reported to stabilize metal oxide NPs, depending on their surface charge by ligand exchange between different aliphatic and phenolic groups on the surface of humic acids, and the ions at the surface of the uncoated or coated ENMs (Yang et al., 2009; Cornelis et al., 2014). Thus, in our study, the dissolved organic matter from the OMES likely makes Ce from nano-CeO₂ more bioavailable to the plants due to better dispersion and stabilization of the particles in the porous media (Conway and Keller, 2016). Zhang et al. (2016) reported that the behavior of nano-CeO₂ in soil and plant strongly depends on soil properties. In addition, the Ce translocation to the aboveground tissues could depend on the presence of Ce ions in the roots (Zhang et al., 2016). However, ionization of the Ce in the plant tissues *in situ*, in response to SOM presence, was not explored in this study. A previous study from the Gardea group has also shown that significantly more Ce is present in the soil solution extracted from nano-CeO₂ spiked soils in presence of higher SOM (Zhao et al., 2012). In contrast to our findings, shoots from corn plants grown in organic soil spiked with uncoated and alginate-coated nano-CeO₂ accumulated significantly lower mg of Ce/kg d wt tissues compared to those grown in unenriched soil (Zhao et al., 2012). The authors suggested

that the carboxylate and phenolate groups in the humus bind to the nano-CeO₂, leading to lower translocation in presence of higher organic matter. This disparity between the previous reports and the current observations could be due to a species-associated response or variations in plant vasculature.

3.4. Impact of nano-CeO₂ on plant growth

The length of the roots and shoots, and the number and area of leaves of plants exposed to 0-500 mg/kg LOMS and OMES are presented in SM Table S3. Plant growth parameters including root and shoot length were not affected across SOM content or nano-CeO₂ exposures. Additionally, the number of leaves per plant was not affected; however, the leaf area was increased significantly ($p \leq 0.05$) only in LOMS plants exposed to 62.5, 250 and 500 mg/kg nano-CeO₂ by 105, 90 and 111%, respectively, compared to control plants. There was no apparent effect on the vegetative growth parameters (roots, stems or leaves) in nano-CeO₂ exposed OMES grown plants.

Due to the evident growth enhancement, antioxidant, and bio-compatible properties of CeO₂, its nanoparticulate form has invited significant attention in agriculture and biomedicine in recent years. Current nanotoxicity studies are more focused on life cycle exposure scenarios (Priester et al., 2012; Zhao et al., 2015; Majumdar et al., 2015) across different types of ENMs and under different environmental conditions (Conway et al., 2015), to benefit their risk assessment in realistic conditions for regulatory measures. Similar to our observations in kidney bean plants, some previous studies have also reported inconsequential effects on overall growth of roots and shoots of cucumber (Zhao et al., 2013), corn (Zhao et al., 2015), and wheat (Du et al., 2015). In tomato plants, Wang et al. (2012) showed no significant effects on the plant length on chronic exposure to 0.1-10 mg/l nano-CeO₂, building upto 130 mg/kg Ce in soil, Barrios et al.

(2015) found a decrease in stem growth at 62.5 and 125 mg/kg nano-CeO₂; however, at higher concentrations, there was no significant difference with respect to control. Priester et al. (2012) also showed decrease in soybean shoot length at 100 and 500 mg/kg nano-CeO₂ exposure in soil. Although in kidney beans there was a slight decrease in shoot length when grown in OMES, but the variation was not significantly different, compared to control plants. As seen in SM Table S3, the increase in leaf cover area in plants grown in nano-CeO₂ amended LOMS could be attributed to beneficial or growth enhancement effects of Ce ions up to certain concentrations (Weiping et al., 2003; Yuguan et al., 2009; Shyam and Aery, 2012; Thomas et al., 2014). However, the leaf count and cover area in OMES plants was unaffected by nano-CeO₂ exposure, similar to previous findings in soybean, corn, and cucumber plants (Priester et al., 2012; Zhao et al., 2013; Zhao et al., 2015) grown in unenriched soils. Due to higher translocation in OMES plants, at all nano-CeO₂ exposure concentrations, the Ce content in OMES leaves was higher than LOMS leaves. Thus, the correlation between leaf growth and Ce content *per se* is debatable. Similar to the kidney bean roots (SM Figure S1), micronutrient contents in the aerial tissues were not affected as shown in SM Table S4 and S5; thus, the expanding leaf cover area in nano-CeO₂ exposed LOMS plants seems not related with the micronutrient content. More studies are needed in order to fully understand the response of plants to nano-CeO₂ exposure in difference soil matrices. Studies utilizing electron microscopy and synchrotron XRF micro-spectroscopy have shown that the Ce taken up by plants is in the form of nano-CeO₂ as well as dissolved Ce (III) ions (Zhang et al., 2012) (Hernandez-Viezcas et al., 2013) (Majumdar et al., 2014). The dissolution of Ce (III) from the NP could occur in the growth media (Schwabe et al., 2015) in the presence of root exudates, soil minerals and SOM, in the root epidermis at point of entry or

inside the plants. Further studies are required to examine how the presence or absence of SOM may affect the speciation of the metals in metal oxide NPs before and after entering plants.

3.5. Impact on photosynthetic pigments, photosynthesis and gas exchange activities

Photosynthesis is a major metabolic process in plants, responsible for capturing light energy for the synthesis of energy producing molecules, and is thus vulnerable to abiotic stress factors (Taiz and Zeiger, 1998). Adverse impact on the photosynthetic pigments in the leaves can affect the regulation of photosynthesis. Photosynthesis is simultaneously associated with other gas exchange activities like transpiration and stomatal conductance, which regulate the plant metabolic processes. The effect of nano-CeO₂ exposure on photosynthetic pigment contents (chlorophyll *a*, chlorophyll *b*, and carotenoids) in the leaves of kidney bean plants grown in LOMS and OMES is given in Figure 4. In LOMS, the effect of nano-CeO₂ on the leaf photosynthetic pigments was inconsequential with the exception of chlorophyll *a*, which increased at all nano-CeO₂ treatments; however, the increase (37%) was statistically significant only at 250 mg/kg nano-CeO₂ exposure concentration (Figure 4A). In contrast, the chlorophyll *a* and carotenoid content in leaves from nano-CeO₂ amended-OMES plants showed a decreasing trend (Figure 4B). In OMES leaves, the chlorophyll *a* decreased by 27 and 18% after 52 days exposure to 125 and 250 mg/kg nano-CeO₂; and, the carotenoid content decreased by 25-28% on exposure to 62.5- 250 mg/kg nano-CeO₂. This indicates that the higher translocation of nano-CeO₂ to the leaves in OMES, leads to reduction in the photosynthetic pigments, which absorb light energy from the sunlight at certain wavelength, and transfer energy in a particular sequence to carry out photosynthesis (Taiz and Zeiger, 1998). However, Ce translocation dependent decrease in photosynthetic pigment did not hold true at the highest exposure concentration in OMES (500 mg/kg nano-CeO₂), where the content of chlorophyll *a* and total carotenoids in the

kidney bean leaves were restored to control levels. Although, the nano-CeO₂ affected the chlorophyll content in the LOMS and OMES leaves, the chlorophyll *a*/chlorophyll *b* ratio was not altered.

Physiological processes including transpiration, stomatal conductance, and net photosynthesis of the LOMS and OMES plants exposed to nano-CeO₂ are shown in Figure 5. In LOMS plants, only at 62.5 mg/kg nano-CeO₂ exposure, the net photosynthesis was increased significantly by 157 %, compared to control (Figure 5C). The simultaneous increase in transpiration rate and stomatal conductance of LOMS plants at 62.5, 250 and 500 mg/kg nano-CeO₂ exposure was statistically insignificant (Figure 5A, B). However, in the OMES plants, at all nano-CeO₂ exposures, the net photosynthesis were significantly higher than the control, but the increase was statistically inconsequential. The stomatal conductance in OMES plants increased significantly at all exposure concentrations (131 ± 17 , 207 ± 27 , 194 ± 14 , 125 ± 32 mmol m⁻² s⁻¹ at 62.5, 125, 250 and 500 mg/kg nano-CeO₂) compared to control plants (31 ± 6 mmol m⁻² s⁻¹). The transpiration rate was also increased from 0.7 ± 0.1 mmol m⁻² s⁻¹ in control plants to 2.4 ± 0.3 , 3.8 ± 0.7 , 3.0 ± 0.1 , and 2.3 ± 0.3 mmol m⁻² s⁻¹ at 62.5, 125 and 250 mg/kg nano-CeO₂ exposures, respectively. As seen in Figure 5, the transpiration rate and stomatal conductance was maximum in the 125 mg/kg nano-CeO₂ exposed OMES plants, which increased by 380 and 207 % than control plants. A significant interaction between the soil type and the nano-CeO₂ exposure concentration was reported for transpiration rate and stomatal conductance.

Similar to our observations in LOMS plants, previous studies on tomato, corn, cucumber and radish have also noted insignificant to slight increase in chlorophyll content on exposure to nano-CeO₂ (Wang et al., 2012; Zhao et al., 2013; Zhao et al., 2015; Barrios et al., 2015; Zh). In

another study, Rico et al. (2015) showed that the chlorophyll content in barley leaves was increased at 125-500 mg/kg nano-CeO₂, similar to our observations in LOMS leaves. The slight increase in the chlorophyll *a* is well correlated with the increase in leaf cover areas in LOMS plants on exposure to nano-CeO₂ (SI Table S3). As shown in many studies, although nano-CeO₂ may cause alterations in some physiological parameters, it does not exert overt toxicity on the plant growth, which could be due to its facile transition between oxidation states (Caputo et al., 2015) acting as a buffer in redox processes or low dissolution (Majumdar et al., 2014; Zhang et al., 2016). Cerium oxide is also used in many parts of the world as fertilizers to improve crop growth and yield. Researchers have shown nano-CeO₂ to initiate protective mechanisms in the plants by upregulation of heat shock proteins (Zhao et al., 2012), or by mimicking the activities of antioxidant enzymes like superoxide dismutase or catalase (McCormack et al., 2014). This also explains the minimum effects on the photosynthetic system and gas exchange in the LOMS plants exposed to nano-CeO₂. Interestingly, the adverse impact on the photosynthetic activities and water transport was much higher in OMES than in LOMS plants exposed to nano-CeO₂. In the OMES plants, the effects on the photosynthetic pigment contents and the photosynthetic activities are contrasting, as these activities are dependent on the pigments to absorb the various wavelengths from sunlight. Our findings are in agreement with previous studies showing reduction in chlorophyll *a* content in wheat plants grown in 100 and 400 mg/kg nano-CeO₂ contaminated field (Du et al., 2015). However, simultaneous increase in the gas exchange activities was interesting to note. As noted in our translocation study, Ce was translocated better in the presence of higher SOM content (Figure 3B). It could be possible that although the nano-CeO₂ decreases the photosynthetic pigments in the leaves, it simultaneously increases the photosynthetic efficiency of the plants. Cerium has been shown to improve the photosynthetic

efficiency of soybean seedlings (Liang et al., 2006). Our observation provide evidences that the alteration in the plant metabolic activities is dependent on the SOM content. As the mechanism of the enhancement in the gas exchange activities in response to nano-CeO₂ is still unknown, further kinetic and competitive studies may be required using photosynthetic enzymes for better clarification.

3.6. Impact of nano-CeO₂ on antioxidant enzyme activities

Figure 6 shows the activities of antioxidant enzymes in roots, stems and leaves in the plants grown in 0-500 mg/kg nano-CeO₂. The APOX and CAT activities in the roots exposed to nano-CeO₂ were not affected. In the stems, CAT activity was increased on exposure to 62.5-500 mg/kg nano-CeO₂ only in LOMS. Additionally, the enzyme activities were not affected in LOMS leaves. However in OMES leaves, CAT activity was significantly increased at 62.5 mg/kg nano-CeO₂ exposure by 177% compared to control (Figure 6B), and APOX activity was increased at 125-500 mg/kg nano-CeO₂ exposure by 465 and 362%, respectively, compared to control (Figure 6A). Differential CAT response in the shoots from kidney bean plants grown in soil with varying SOM contents validates that the CAT mimetic activity of CeO₂ is enhanced with more SOM in the growth media, due to stabilization of CeO₂ before entering the plants. In agreement with our observations in roots and leaves, Barrios et al. (2015) found no effect on CAT activities in tomato plants exposed to pristine nano-CeO₂; however the CAT activity in the plants was increased upon exposure to more stable citric acid-coated CeO₂. In barley leaves, CAT activity was not affected, but APOX activity increased significantly at 125-500 mg/kg nano-CeO₂ exposure in soil (Rico et al., 2015). In another recent study, Ma et al. (2016) reported that CAT or superoxide dismutase activity in the *Brassica rapa* plants was not affected by every 2 day chronic addition of fixed volume (20ml) of 10 and 1000 mg/L nano-CeO₂ for 17 days (net addition of 32 and 320 mg/kg nano-CeO₂, respectively). In accordance with our supporting evidence on the differential response to nano-CeO₂ exposure with varying SOM content, the

stress response in kidney bean plants grown in OMES was thus more pronounced than in LOMS. In the OMES plants, although the CAT was responsible to combat stress in the leaf tissues at 62.5 mg/kg nano-CeO₂ exposure, the APOX acted as the primary antioxidant enzyme to fight the stress from 125-500 mg/kg nano-CeO₂ exposures.

4. Conclusions

Among other soil properties, natural organic matter influences the behavior of nanoparticles in the soils as well as their effects on plants. This study clearly states that enhancement of organic matter in the soil increases the mobility of the nano-CeO₂ within the plants. This leads to easier access of the nano-CeO₂ to the metabolic machinery of the plants, which alters physiological activities in the leaves pertaining to food and energy production and plant health. On the other hand, in natural sandy loam soil with low organic matter, the nano-CeO₂ aggregated or attached to soil grain surface due to opposite charge, and thus restricted primarily to the below ground biomass. This study suggests that fate, bioavailability and biological effects of nano-CeO₂ are highly dependent on the properties of the complex soil matrix. Further investigations are required in the rhizospheric region to understand how the soil physico-chemical properties influence the chemistry between nano-CeO₂, plant roots and soil components. This may help in a better understanding of the mechanisms by which the nano-CeO₂ affect plant health and major metabolic functions.

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Figure captions

Figure 1. Concentration of cerium (mg/kg) in low organic matter soil (LOMS) and organic matter enriched soil (OMES) amended with 0-500 mg/kg nano-CeO₂ before sowing kidney bean seeds. LOMS and OMES are sieved and refer to particles ≤ 2 mm. OMES-OM refer to the sieved organic matter fraction (>2 mm) of the OMES. Values are expressed as mean $\pm$ SE ($n = 4$). Bars with different letters within same soil type, represent significant difference at $p \leq 0.05$.

Figure 2. Concentration of cerium (mg/kg) in three soil zones: surface, rhizosphere, and bottom zones of low organic matter soil (LOMS) and organic matter enriched soil (OMES) after 52 days of exposure to 0-500 mg/kg nano-CeO₂ in presence of kidney bean plants. Values are expressed as mean $\pm$ SE ($n = 4$). No significant differences were observed at $p \leq 0.05$ between three zones in each soil treatment.

Figure 3. Cerium accumulation in kidney bean plant (A) roots, and (B) leaves, exposed to 0-500 mg/kg nano-CeO₂ in low organic matter soil (LOMS) and organic matter enriched soil (OMES). Values are expressed as mean $\pm$ SE ($n = 4$). Bars with different letters represent significant difference at $p \leq 0.05$ within a soil type.

Figure 4. (A) Photosynthetic pigment contents ($\mu\text{g/ml}$) in LOMS leaves, (B) Photosynthetic pigment contents ($\mu\text{g/ml}$) in OMES leaves of plants exposed to 0-500 mg/kg nano-CeO₂. Values are expressed as mean $\pm$ SE ($n = 4$). Different letters represent significant difference at $p \leq 0.05$ within a soil type.

Figure 5. (A) Transpiration rate ($\text{mmol m}^{-2} \text{s}^{-1}$), (B) Stomatal conductance ($\text{mmol m}^{-2} \text{s}^{-1}$), and (C) Net photosynthesis ($\mu\text{mol m}^{-2} \text{s}^{-1}$) in leaves of plants grown in low organic matter soil (LOMS) and organic matter enriched soil (OMES) amended with 0-500 mg/kg nano-CeO₂. Values are expressed as mean $\pm$ SE ($n = 4$). Bars with different letters represent significant difference at $p \leq 0.05$ within a soil type. The symbol * represent significant interaction between soil and nano-CeO₂ exposure concentration at $p \leq 0.05$.

Figure 6. (A) Ascorbate peroxidase and (B) Catalase activity ($\mu\text{mol H}_2\text{O}_2$ degraded $\text{min}^{-1} \text{mg}^{-1}$ FW) in root, stems, and leaf tissues of plants grown in low organic matter soil (LOMS) and organic matter enriched soil (OMES) amended with 0-500 mg/kg nano-CeO₂. Values are expressed as mean $\pm$ SE ($n = 4$). Bars with different letters represent significant difference at $p \leq 0.05$ within a soil type.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Table 1. Low organic matter soil (LOMS) and organic matter enriched soil (OMES) properties in response to 0-500 mg/kg nano-CeO₂.

Soil	Low organic matter soil (LOMS)					Organic matter enriched soil (OMES)				
nano-CeO ₂ treatment (mg/kg soil)	0	62.5	125	250	500	0	62.5	125	250	500
Soil class	sandy loam soil (64% sand, 31% silt, 5% clay)					sandy loam soil (64% sand, 31% silt, 5% clay)				
Loss on ignition organic matter (%)	4.2					10.1				
Bulk density (g/cm ⁻³)	1.11					0.83				
pH (water)	7.92 ± 0.08a	7.98 ± 0.20a	7.88 ± 0.05a	7.68 ± 0.02a	7.95 ± 0.03a	7.28 ± 0.05a	7.00 ± 0.07a	6.90 ± 0.04a	6.83 ± 0.02a	6.98 ± 0.02a
pH (CaCl ₂)	7.53 ± 0.05a	7.60 ± 0.04a	7.68 ± 0.02a	7.65 ± 0.03a	7.70 ± 0.00a	7.05 ± 0.03a	7.03 ± 0.05a	6.93 ± 0.02a	7.13 ± 0.05a	6.98 ± 0.03a
Electrical conductivity	207.5 ± 4.8a	395.0 ± 60.8b	472.5 ± 11.1bc	545.0 ± 38.6c	470.0 ± 9.1bc	485.0 ± 35.7a	947.5 ± 21.7b	992.5 ± 19.7b	972.5 ± 34.5b	982.5 ± 32.2b
Total dissolved solid	92.5 ± 2.5a	190 ± 31.9b	227.5 ± 4.8b	262.5 ± 18.9b	230.0 ± 4.1b	240.0 ± 19.2a	470.0 ± 10.8b	492.5 ± 9.5b	482.5 ± 17.0b	497.5 ± 20.6b
Cation exchange capacity (meq/100g)*	23.3 ± 1.8a	21.6 ± 1.5a	26.2 ± 3.2a	23.8 ± 0.9a	22.4 ± 1.0a	33.13 ± 1.3a	34.16 ± 0.6a	36.9 ± 1.9a	34.4 ± 0.9a	34.1 ± 1.2a
<u>Macro-nutrients:</u>										
Total kjeldahl N (%)*	0.06 ± 0.002a	0.07 ± 0.001a	0.07 ± 0.001a	0.07 ± 0.000a	0.07 ± 0.002a	0.09 ± 0.004a	0.10 ± 0.002a	0.11 ± 0.001a	0.11 ± 0.001a	0.11 ± 0.002a
Potassium (mg/kg)	4915 ± 147a	4750 ± 162a	4720 ± 162a	5052 ± 58a	4962 ± 169a	5980 ± 262ab	5864 ± 130ab	6857 ± 179a	5602 ± 353ab	5284 ± 159b
Phosphorus (mg/kg)	910 ± 5.7a	919 ± 9ab	908 ± 8ab	929 ± 5b	921 ± 10b	846 ± 30a	840 ± 4a	902 ± 45a	788 ± 32a	789 ± 39a
Calcium (mg/kg)	28595 ± 331a	28870 ± 166 a	28707 ± 206a	26565 ± 173b	26751 ± 217b	27753 ± 992a	27509 ± 95a	28771 ± 239a	26414 ± 660a	25966 ± 633a
Magnesium (mg/kg)	6532 ± 16a	6577 ± 112a	6519 ± 60a	5991 ± 45b	6026 ± 66b	6151 ± 249ab	6086 ± 50ab	6474 ± 65a	5888 ± 186ab	5663 ± 119b
Sulfur (mg/kg)*	706 ± 25a	820 ± 10b	828 ± 24b	764 ± 14ab	732 ± 10a	779 ± 42a	905 ± 6b	958 ± 19b	890 ± 35ab	863 ± 18ab
<u>Micronutrients:</u>										
Aluminum (mg/kg)	21094 ± 70a	20901 ± 834a	20642 ± 360a	20169 ± 228a	20045 ± 387a	20887 ± 783ab	21158 ± 233ab	22962 ± 348a	19509 ± 1010b	18531 ± 649b
Copper (mg/kg)	24.3 ± 0.2a	23.5 ± 0.1b	23.7 ± 0.2ab	14.0 ± 0.1c	13.8 ± 0.2c	24.4 ± 1.4a	23.7 ± 0.3a	25.0 ± 0.4a	23.5 ± 0.7a	22.1 ± 0.5a
Iron (mg/kg)*	17371 ± 89a	17434 ± 220a	17453 ± 128a	14370 ± 90b	14457 ± 139b	14329 ± 453ab	14203 ± 122ab	15063 ± 162a	13768 ± 326ab	13388 ± 255b
Manganese (mg/kg)	342 ± 4a	362 ± 6a	342 ± 4a	312 ± 1a	332 ± 3a	376 ± 22ab	349 ± 3ab	369 ± 5a	332 ± 9ab	324 ± 8b
Nickel (mg/kg)	8.0 ± 0.1a	7.6 ± 0.1a	7.9 ± 0.1a	7.4 ± 0.4a	7.6 ± 0.3a	8.4 ± 0.9a	6.9 ± 0.3a	7.8 ± 0.2a	6.8 ± 0.7a	6.3 ± 0.1a
Zinc (mg/kg)	64.6 ±	64.4 ± 3.0a	60.9 ± 2.3a	44.0 ±	43.7 ±	55.3 ± 3.1a	51.2 ± 1.1ab	54.4 ±	48.4 ± 2.0ab	46.2 ±

	4.1a	0.3b	0.7b	0.9ab	1.0b
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Values are expressed as mean $\pm$ SE ($n=4$). The mean values with different alphabetical letters within each soil type denote significant difference at $p \leq 0.05$ detected by one-way ANOVA followed by post-hoc Tukey's test. Parameters with * represent significant difference between untreated LOMS and OMES detected by paired t-test (two-tailed).