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The Short Exposure effect of CeO_2 Nanoparticles on Microbial Metabolism in Agricultural Soil

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Abstract: With the development of nanotechnology, the soil ecosystem is becoming increasingly exposed to engineered nanoparticles (NPs). Few studies thus far have reported the susceptibility of soil microorganisms to NPs. The aim of this work is to understand the effects of CeO_2 NPs on microorganism activity and to evaluate the nanotoxicity in agricultural ecosystems. We incubated soil samples contaminated with CeO_2 NPs at concentrations of 0, 0.5, 5, 50 mg kg^{-1} for 30 days. We quantified the toxic effect of CeO_2 NPs on soil metabolism by combining microcalorimetry with specific enzymatic tests (urease, catalase and fluorescein diacetate hydrolase, FDA) and azobacter counting. The thermodynamic parameters obtained from the power-time curves show reductions of total heat output, Q_{total}, and peak heat output, P_{max}, with increasing CeO_2 NP concentration. This finding reveals that CeO_2 NPs are toxic to the metabolism of microbial populations in soil. Similar tests with urease, catalase and FDA, exhibited similar negative concentration relationships, thus providing further evidence to support the microcalorimetric results. Furthermore, the soil diazotroph group is particularly sensitive to CeO_2 NPs concentrations of 5 and 50 mg kg^{-1}, indicating disturbance of N cycling. In conclusion, these results indicate that CeO_2 NPs become bioavailable for microorganisms in soil, thereby exerting toxic effects on metabolism activity and the azobacter group.

Keywords: *CeO_2 NPs, Soil microorganism metabolism, Enzyme activity, Toxicity*

1. Introduction

Nanotechnology has the potential to dramatically promote and improve agricultural production in application of nano-fertilizer, nano-pesticide, nano-herbicide. It has previously been shown that engineered nanoparticles (NPs) are inevitably introduced into soil matrix through biosolids [1]. However, there are currently large uncertainties associated with the knowledge of fate and behavior in agricultural systems. Moreover, estimation of environmental contaminants is challenging, because grave damage to nontarget organisms is hard to diagnose and control [2]. Phytotoxicity results have shown that CeO_2 NPs affect various plant metabolism including seed germination, photosynthesis rate, chlorophyll content, food quality and genotoxicity [3-7]. Recent studies have shown that these particles exhibit an extremely negative effect through a bioluminescence test [8], and are capable of altering the protein and polysaccharide structure of extracellular polymeric substances in *Sinorhizobium meliloti* [9]. The bacteriostatic effects of CeO_2 NPs necessitate understanding toxicity of CeO_2 NPs on microorganisms in the soil environment, because the bioavailability of NPs may depend on their retention and soil properties. However, knowledge of the fate and behavior of CeO_2 NPs in the soil environment is still very limited. A previous study revealed that dissolution of CeO_2 NPs is low in soil at pH values of 7 and 9 [10]. It can be considered that CeO_2 NPs will remain stable in the soil environment for an extended period of time. It was reported that free Ce were found in soil contaminated with CeO_2 NPs, indicating it may be up taken by microorganisms [11].

Any change in microorganisms that involve organic decomposition, nutriment cycling, plant protection and symbiosis can influence soil health and fertility [12]. Soil health can be indicated by the abundance and activity of the microbial community. Soil fertility is crucial for plant growth and food production since most food consumed by humans and animals is grown in soil [13]. However, few studies have assessed the impact of CeO_2 NPs on the susceptibility of functional microorganism such as azobacter in soil. Azobacter promotes plant growth and benefits nitrogen cycling in soils, and may be used to evaluate of nanotoxicity on microbial biodiversity and community structure conservation [14]. This motivates the need to better understand the behavior of CeO_2 NPs in soil and their interactions with microorganisms especially under intensive cultivation. The purpose of this work is to provide new data to understand the effect of CeO_2 NPs on microorganisms in arable soil. The experiments presented in this work identify the potential toxicity of CeO_2 NPs on azobacter and quantify overall soil microbial activity, including the metabolism of thermogenesis and soil enzyme activities. The thermodynamic technique in conjunction with other specific bio-tests have proven to be useful in measuring the toxicity of

environmental hazardous pollutants on soil microbial metabolism [15, 16]. Soil enzyme activities are excellent indicators of soil microbial function and nutrient cycling [17]. As such, this study focuses on investigating the toxicity of CeO_2 NPs on microbial metabolism in arable soil. We systematically measure the metabolic thermogenic flux, enzyme activities (urease, catalase, fluorescein diacetate hydrolase) and number of soil azobacter as markers of soil fertility and soil health.

2. Material and methods:

2.1. Experiment design

This study makes use of uncontaminated soil collected from arable maize cropland in Hebei Province of China (38°46'51", 115°33'36"). Soil samples were collected at a depth of 5-10 cm after removal of the top surface layer. Samples were passed through a 2 mm sieve to separate roots and large particles. The physicochemical properties of are: pH 7.46, organic matter content 16.8 g kg^{-1}, total N 0.82 mg kg^{-1}, available P 13.3 mg kg^{-1} and available K 96.43 mg kg^{-1}.

The CeO_2 NPs was with a purity >99.9% and surface area of 50-60 m^2 g^{-1}. Transmission electron microscopy was used to verify that CeO_2 NPs have an average particle size of 10-20 nm (Figure 1). Before addition to the test-microcosm, CeO_2 NPs were diluted in distilled water and sonicated for 30 min to achieve a homogeneous mixture [18]. Each microcosm consists of 100 g of soil in sterile plastic bottle. These CeO_2 NP suspensions were dropped into the soil and stirred for at least 35 min to achieve concentrations of 0.5, 5.0 and 50.0 mg kg^{-1}, respectively. A microcosm free of CeO_2 NPs was used as the control. All treatments were prepared in triplicate. After adding the CeO_2 NPs, the soil was incubated at 25 ºC for 30 days. The soil water content was then adjusted to 70% of water holding capacity.

2.2. Metabolism of soil microorganisms

The TAM III multi-channel microcalorimeter (TA instruments, New Castle, DE, USA) was used measure the metabolism of microorganism in soil after one month of incubation. This microcalorimeter was equipped with 12 channels allowing continuous monitoring of the microbial metabolism activity. The 4.5 mL stainless steel ampoules were sterilized before being used in the experiment. Every measurement of thermal metabolism was carried out in ampoules containing 1.0 g of soil and 200 µL of solution containing 5.0 mg of glucose and 5.0 mg of ammonium sulfate to support the growth of soil microorganism [19]. In the exponential growth phase, the relationship between microbial number (n) and growth rate constant (k) follows the equation [20].

$$n = n_0 e^{kt} \quad (1)$$

Where n_0 is the initial soil microorganism number and k is the growth rate constant. Heat production, Q, evolving in the ampoule under limited nutrition source follows the same relationship above [19,21,22].

$$Q = Q_0 e^{kt} \quad (2)$$

Similarly, the thermal power output, p_t, which is equal to the first time derivative of Q, also obeys the same kinetics:

$$p_t = p_0 e^{kt}; \quad \ln p_t = \ln p_0 + kt \quad (3)$$

If the NPs have a toxic effect, they would reduce microbial biomass and this effect would be reflected by a smaller value of k. We calculate the total thermal effect, Q, the microbial growth rate constant, k, and the peak-time value, P_{max} at time T_{max}, from power-time curves for all curves. These parameters can be used as indices to evaluate how fast glucose is decomposed by soil microbes because metabolic rate is proportional to glucose degradation rate [23, 24].

2.3. Soil enzyme activity and azobacter counting

The activity of urease is measured using colorimetric analysis of ammonium released from urea hydrolysis. In this procedure, 2.5 g of soil samples added in 50 mL Erlenmeyer flasks were mixed with 0.5 mL toluene for 15 min. Then, 2.5 mL of 10% urea and 5 mL of citrate buffer (pH 6.7) were added to the samples and incubated at 37 °C for 24 h. After incubation, the mixtures were diluted to 50 mL with 37 °C distilled water and oscillated thoroughly. 3 mL filtrate was transferred into a 50 mL volumetric flask, to which 10 mL of distilled water, 3 mL of 1.35 mol L^{-1} sodium phenate and 3 mL sodium hypochlorite (active chlorine 0.9%) were added. Urea hydrolysis was subsequently determined by absorbance of the supernatant at 578 nm.

Catalase activity was determined by back-titrating residual H_2O_2 with 0.1 mol L^{-1} $KMnO_4$ solution [25]. A 40 mL aliquot of distilled water and 5 mL of 0.3% H_2O_2 were added to 5 g of soil. The mixture was shaken for 30 min, and 5 mL of 1.5 mol L^{-1} H_2SO_4 was then added to terminate the reaction. A 25 mL aliquot of the filtered solution was titrated with 0.01 mol L^{-1} $KMnO_4$. An identical mixture without the inclusion of H_2O_2 was used as the control.

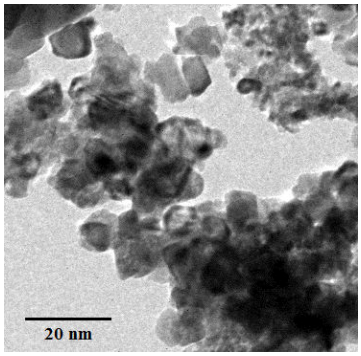


Figure 1*. Transmission electron microscopy (TEM) images of CeO_2 NPs*

Fluorescein diacetate (3', 6'-diacetylfluorescein; FDA) hydrolase was carried out at 490 nm as absorption of the hydrolysis product fluorescein following [26]. A 5 g sample of soil and 15 mL of 60 mM Potassium dihydrogen phosphate buffer (pH 7.6) were added to a 50 mL Erlenmeyer flask and shaken at 30ºC. After 30 min, 0.2 mL of 1000 μg ml^{-1} of FDA solution was added, and the suspension was shaken for an additional 30 min. The reaction was terminated by adding a 15 mL of 2:1 chloroform/methanol mixture. The suspension was centrifuged, and the absorbance of the supernatant was measured at 490 nm.

Soil samples (5.00 g) were placed in Erlenmeyer flasks containing sterile water and shaken (180 rpm, 30 min). This was followed by continuous dilutions for plate counting. Viable counts of cultural azobacter were performed on Ashby Mannitol Phosphate Agar (mannitol 10 g, KH_2PO_4 0.2 g, NaCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCO_3$ 5 g, $CaSO_4$ 0.1 g, agar 15 g L^{-1}, pH7.0), and incubated at 28ºC for 7 days. All bioassays were conducted with materials that are in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

2.4. Data analysis

SPSS 13.0 (SPSS International, USA) was used for statistical analysis. Differences between each treatment and the control were tested by One-Way ANOVA. The Duncan test was applied to determine significant differences at the level $p<0.05$. Correlation analyses ($p<0.05$) between the parameters were performed with two-tailed Pearson tests.

3. Results and discussion:

3.1. The effect of CeO_2 NPs on metabolic activity

Figure 2 shows the thermograms of soil samples spiked with various concentrations CeO_2 NPs. Over the course of 40 h, soil heat effluxes exhibit a similar pattern, reaching a peak after ~10 h and declining gradually thereafter. However, the curves differ in their growth characteristics. Namely, the height of each peak decreases with increasing concentration of CeO_2 NPs. The microcalorimetric parameters of soil at different CeO_2 NP concentrations are shown in Table 1. The global maxima (P_{max}) occur at earlier times (T_{max}) for samples contaminated with higher CeO_2 NPs concentrations, with the control exhibiting its peak at the latest time. All differences are significant ($p<0.05$). It is also noteworthy that P_{max} values decrease with increasing concentrations of CeO_2 NPs; P_{max} of the control is as high as 913.04 μW (curve a), exceeding the treatment of 50 mg/kg CeO_2 NPs by 249.71μW (curve d).

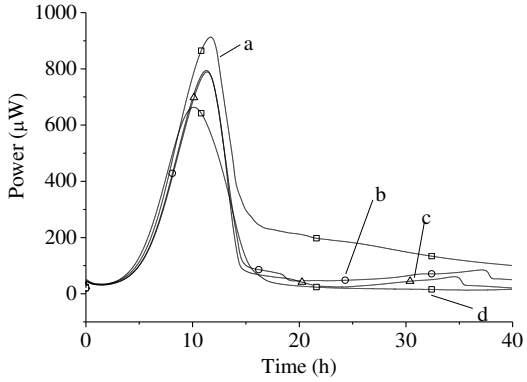


***Figure 2**. Thermogenesis metabolism curves of soil microorganism spiked with CeO_2 NPs. (a) control; (b) 0.5 mg kg^{-1}; (c) 5 mg kg^{-1}; (d) 50 mg kg^{-1}*

These results clearly indicate that agricultural soil contaminated with CeO_2 NPs produce substantial changes to the metabolic activity of soil microorganisms. This is implied by the decrease in the total heat evolution, Q_{total}, with increasing concentrations of CeO_2 NPs ($p<0.05$). Since the same amounts of glucose and ammonia sulphate were added to each ampoule, differences in the inhibition of metabolic activity can be solely attributed to the toxicity of CeO_2 NPs.

In the microenvironment of ampoules, oxygen and nutrients are limiting factors for the growth and reproduction of microorganisms. At the biochemical level, heat output from soil samples show a 1:1 molar relationship with substrate addition. For this reason, the Q_{total} can represent the apparent degradation of the substrate. Therefore, Q_{total} can be considered a reliable indicator expressing minor changes of many microbial populations in response to various environmental stress factors. The change of the growth rate constant and the maximum heat production rate of each experiment indicated that CeO_2 NPs are capable of changing the ecological niche of soil microorganisms. Several comparisons of the kinetic parameters suggest that CeO_2 NPs have a major effect on soil microbial activity. Thermogenesis involves many enzymes that drive glycolysis, the Krebs cycle and electron transport train. This variability in the microbial activity of soil might be caused by direct oxidative stress on enzymes or released metal ions from nanoparticles [27].

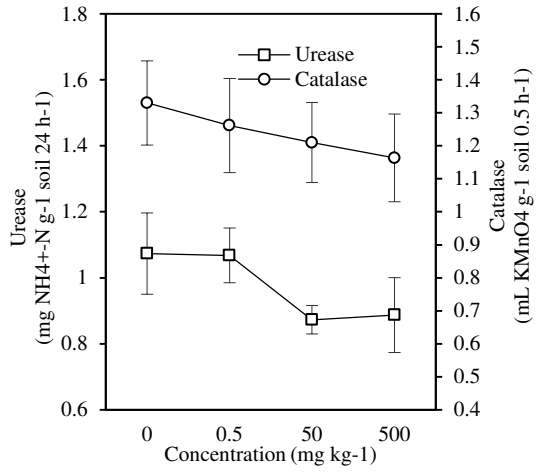
***Table 1**. Microcalorimetric parameters of soil treated with different concentration of CeO_2 NPs*

Dose (mg kg^{-1})	Q_{total} [a] (J g^{-1})	T_{max} [b] (h)	P_{max} [c] (μW)	k [d] (h^{-1})
Control	35.63 ±1.32 A	11.69 ±0.120 A	913.04 ±4.58 A	0.51 ±0.125 A
0.5	22.63 ±1.26 B	11.28 ±0.098 B	794.05 ±3.69 B	0.46 ±0.095 A
5	20.20 ±2.16 B	11.35 ±0.085 B	789.21 ±5.25 B	0.47 ±0.147 A
50	18.58 ±1.65 C	10.13 ±0.124 C	663.33 ±7.27 C	0.50 ±0.084 A

Data are means of three replicates ± SD (standard deviation), cluster labeled with the different alphabet indicates statically significant difference ($p<0.05$, Duncan test)
[a] The total heat output calculated from the Power-time curve.
[b] The time to reach the maximum of the peak.
[c] The power at the maximum of the peak.
[d] The microbial growth rate constant.

3.2. The effect of CeO_2 NPs on soil enzyme activities

Urease, catalase and FDA hydrolase activities for soil contaminated with CeO_2 NPs for one month are shown in Figure 3. Urease activity is widely distributed in soil and approximately 17-77% of bacteria and 78-98% of fungi in soil have the capacity to catalyze the conversion of urea and amine to ammonia. In agreement with the results shown for the thermograms, Fig. 3 shows that lower urease activity is associated with higher CeO_2 NPs concentrations. It is well known that catalase accounts for biodiversity in soil environment. CeO_2 NPs affect soil catalase activity. By contrast, FDA was statistically lower than the control at all CeO_2 NPs concentrations. FDA is a good indicator of overall soil microbial activity as it is involved in metabolic processes of organic matter transformation in order to gain energy for the growth of microbes. Soil enzyme activities are often used as direct indicators of soil metabolic demand and fertility. It has been suggested that maintaining critical functions may ultimately be more important than maintaining taxonomic diversity in soil microbial communities [28]. Reduction in soil enzyme activity through toxic contamination effects on soil micro flora, are a reliable indicator of the current microbial biological state. A bacterial cell usually contains approximately 1000 different enzymes, many of which are associated with the cell membrane. Thus, one possible reason for this reduction could be CeO_2 NP-induced cell membrane damage [29]. ZnO NPs can also inhibit soil protease, catalase, dehydrogenase, phosphatase and peroxidase activities [17, 25].



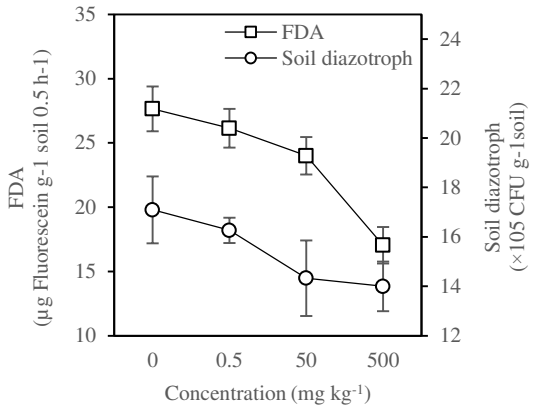


***Figure 3**. Enzyme activities and the colony forming unit of soil treated with CeO_2 NPs*

3.3. Viable counting of soil diazotroph

Soil diazotrophs are important functional bacteria in nitrogen enrichment for plant growth as promoters of nitrogen fixation and stimulators of the N-cycle in soils, and are also good proxies for assessing soil ecosystem fertility and soil health. It was estimated that soil azobacters can provide 10-50% of the total N requirement for the growth of wheat [30, 31]. Furthermore, these bacteria may produce phytohormones, phosphorous for plant growth to promote nutrient uptake and improve water conditions and stress resistance to plant pathogens [32, 33]. We measured the abundance of azobacter via Ashby agar counting (Figure 3). The colony forming unit (CFU) of diazotroph in the control was greater than 0.5 mg kg^{-1} CeO_2 NPs, 5 mg kg^{-1} CeO_2 NPs and 50 mg kg^{-1} CeO_2 NPs. Significant reduction was only observed in soil contaminated with 5 mg kg^{-1} and 50 mg kg^{-1} of CeO_2 NPs relative to the control ($p<0.05$).

The number of diazotroph correlates positively with the thermal metabolism parameters and enzyme activities. Our results indicated a tight link between thermogenesis and microorganism activity. Many studies reported that soil enzyme activities are positively correlated with microbes counts [34]. Certain microorganisms are more susceptible to the stress of CeO_2 NPs than others. Agricultural soil contaminated with ZnO and TiO_2 NPs resulted in a decrease of N-fixation capacity of the microorganism, *Bradyrhizobium japonicum* [35]. Many results also reported other NPs exhibit toxic effects on N-cycling bacteria and N-fixation rate of aquatic organism [36, 37].

4. Conclusion:

In summary, the present data show that CeO_2 NPs potentially have negative effects on the microorganism activity of agricultural soil. We employed calorimetry and indicators of soil fertility, i.e., urease, catalase, FDA, and azobacter to develop an efficient and fast screening assay. Our results successfully demonstrated that the toxic effect of

CeO_2 NPs strongly depends on their concentration. We also used thermodynamic parameters as reliable indicators to reflect the negative effect of CeO_2 NPs on soil microorganism activity. Analysis of the three soil activities, can allow us to better understand how CeO_2 NPs may interfere with soil fertility. We find that soil diazotroph is very sensitive to CeO_2 NPs at concentrations of 5 and 50 mg kg^{-1}. Further assays need to be conducted to test the susceptibility and diversity of key bacteria groups such as rhizobium, ammonifier, nitrifier, etc. These functional bacteria can pave the way for improved knowledge of the ramifications of nanotoxicity on nutrient cycling and ecological service.

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