RESPONSE OF SOIL MICROBIAL BIOMASS TO CeO_2 NANOPARTICLES

RÉPONDUE DE LA BIOMASSE MICROBIENNE DU SOL AUX NANOPARTICULES DE CeO_2

RISPOSTA DELLA BIOMASSA MICROBICA DEL SUOLO ALLE NANOPARTICELLE DI CeO_2

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Abstract

Aim of this work was to assess the impact of the chronic exposure of CeO_2 nanoparticles (NPs) (50 to 105 nm nominal size) on soil microbial biomass.
To evaluate if the CeO_2 NPs can affect the soil quality, they were mixed to an A1 and A2 horizon of Epileptic Cambisols at a concentrations of 100 ppm and incubated in lab for short and medium (7 and 60 days) times, at a constant temperature (25°C) and moisture (60% WHC).
The preliminary results of the soil physicochemical analyses have showed an insolubility of the CeO_2 NPs at short-term incubation in water, EDTA and aqua regia. The biological assays detect a storing of Ce-CeO_2 in the microbial biomass at short time that decreases in the C amount. An increment of the basal respiration and a decrease in the amount of carbon soil microbial biomass determined a higher metabolic quotient (qCO_2) than the control test, that identifies a stressful situation, most evident in the short term condition.
Physical-chemical characterization of the CeO_2 NPs and of the soil before and after the NPs addition, was carried out by means of Environmental Scanning Electron Microscope (ESEM) and an Energy Dispersive Spectroscopy (EDS). The investigations showed Ce-NPs and Ce-compounds in both- incubation-condition samples. The control soil showed the presence of cerium associated with other elements, like P, Nd, La, Th e Si. From literature, it appears that these elements identify Monazite-Ce/Nd minerals, whose chemical formulas are respectively (Ce, La, Nd, Th) PO_4 and (Nd, Ce, La) (P, Si) O_4. The presence of CeO_2 NPs was clearly detected in soil and recognized by ESEM morphological observations coupled with EDS characterization. The NPs chemical composition appears unaltered, while the size can be modified by NPs aggregation and clustering.
The results contribute to setting reference baseline values of cerium in soil and indicate an impact on the amount of carbon soil microbial biomass due to a higher metabolic quotient (qCO_2) that can condition the soil fertility.

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Keywords: *nanoparticles; cerium oxide; bioindications; soil microbial biomass; electron microscopy.*

Résumé

But de ce travail a été d'évaluer l'impact d'une exposition chronique de nanoparticules de CeO_2 (NP diamètre compris entre 50 et 150 nm), sur la biomasse microbienne du sol. L'évaluation de l'effet qui peuvent avoir sur la qualité du sol a été expérimentée sur horizons A1 et A2 d'Epileptic Cambisols en utilisant concentrations de 100 ppm avec des incubations en laboratoire pour un bref et une période moyenne de temps, 7 et 60 jours, respectivement, en maintenant température constante, 25°C et humidité, 60% WHC. Les résultats préliminaires des analyses physique et chimiques du sol effectués avec l'eau, EDTA et aqua regia ont souligné une insolubilité des NP - CeO2 après incubation pour une brève période. L'essai biologique a souligné dans la brève période une accumulation de Ce-CeO_2 en la biomasse microbienne et un décrément du contenu en C organique soluble. L'accroissement de la respiration basale et le décrément du contenu en carbone de la biomasse microbienne ont induit un quotient métabolique élevé (qCO_2) respect au test de contrôle, tel à souligner une situation de stress très évident dans les conditions de brève période. La caractérisation chimique-physique des nanoparticules de CeO_2 et du sol avant et après l'addition des mêmes NP, elle a été effectuée en microscopie électronique (ESEM *Scanning Electron Microscope),* jumelée à spectroscopie aux rayons X (EDS *Energy Dispersives Spectroscopy*). L'enquête a souligné la présence de nanoparticules de Ce et de mélanges de Ce en les deux les incubations des champions. Le sol de contrôle a montré présence de Ce associé aux autres éléments quel P, Nd, Vous, Th et La; de la littérature on déduit que tels éléments identifient minéraux de monazite-Ce/Nd à la formule chimique, (Ce,La,Nd,Th)PO_4 et (Nd,Ce,La)(P,Si)$_{O4}$. La présence de nanoparticules de Ce a été déterminée et reconnue par les observations morphologiques dans le champion de sol avec de l'ESEM complété par la caractérisation avec de l'EDS; on est pu, donc, souligner comme la composition chimique des nanoparticules soit resté inaltéré, pendant qu'elle se soit modifiée la dimension en fonction de leur agrégation. Les résultats ont contribué à mettre au point les valeurs de fond de Ce dans le sol et la présence de nanoparticules il a souligné un impact sur la quantité de C de la biomasse microbienne dûe à un quotient métabolique élevé à conditionner la fertilité naturelle du sol.
Mots clés: *nanoparticules; oxyde de cérium; bioindicateurs; biomasse microbienne du sol; microscopie électronique*

Riassunto

Scopo di questo lavoro è stato quello di valutare l'impatto di una esposizione cronica di nanoparticelle di CeO_2 (NP, diametro compreso tra 50 e 150 nm) sulla biomassa microbica del suolo. La valutazione dell'effetto che NP di CeO_2 possono avere sulla qualità del suolo è stata sperimentata su orizzonti A1 e A2 di Epileptic

Cambisols utilizzando concentrazioni di 100 ppm con incubazioni in laboratorio per un breve e un medio periodo di tempo (7 e 60 giorni, rispettivamente) mantenendo costanti temperatura (25°C) ed umidità (60% WHC). I risultati preliminari delle analisi fisico-chimiche del suolo effettuate in acqua, EDTA ed aqua regia hanno evidenziato una insolubilità delle NP- CeO_2 dopo incubazione per un breve periodo. Il saggio biologico ha evidenziato nel breve periodo un accumulo di Ce-CeO_2 nella biomassa microbica ed un decremento del contenuto in C organico solubile. L'incremento della respirazione basale ed un decremento del contenuto in carbonio della biomassa microbica hanno indotto un elevato quoziente metabolico (qCO_2) rispetto al test di controllo, tale da evidenziare una situazione di stress molto evidente nelle condizioni di breve periodo. La caratterizzazione chimico-fisica delle nanoparticelle di CeO_2 e del suolo prima e dopo l'aggiunta delle NP stesse, è stata effettuata in microscopia elettronica ambientale (ESEM *Scanning Electron Microscope*) accoppiata a spettroscopia a raggi X (EDS *Energy Dispersive Spectroscopy*). L'indagine ha evidenziato la presenza di nanoparticelle di Ce e di composti di Ce in entrambe le incubazioni dei campioni. Il suolo di controllo ha mostrato presenza di Ce associato ad altri elementi quali P, Nd, La, Th e Si; dalla letteratura si evince che tali elementi identificano minerali di monazite-Ce/Nd a formula chimica $(Ce,La,Nd,Th)PO_4$ e/o $(Nd,Ce,La)(P,Si)O_4$. La presenza di nanoparticelle di Ce è stata determinata e riconosciuta dalle osservazioni morfologiche nel campione di suolo con ESEM integrate dalla caratterizzazione con EDS; si è potuto, quindi, evidenziare come la composizione chimica delle nanoparticelle sia rimasta inalterata, mentre si sia modificata la dimensione in funzione della loro aggregazione. I risultati hanno contribuito a mettere a punto i valori di fondo di Ce nel suolo e la presenza di nanoparticelle ha evidenziato un impatto sul quantitativo di C della biomassa microbica dovuto ad un più elevato quoziente metabolico tale da condizionare la fertilità naturale del suolo.
Parole chiave: *nanoparticelle; ossido di cerio; bioindicatori; biomassa microbica del suolo; microscopia elettronica*

Introduction

Nanoparticles are entities that are defined with a size range between 1 and 100 nm. They can be engineered, or unintentionally generated by industrial combustive processes or of natural origin (geogenic, biogenic, atmospheric). The production of engineered nanoparticles (NPs) is growing day by day because, thanks to their physical and chemical properties, are used in different sectors, from cosmetics to textile industry, from medicine to agriculture, from the automotive industry to food. The cross-use of these compounds can determine an unintentional pollution of various environmental compartments especially at the end of the life cycle, and a following accumulation in various environmental compartments. Their effect on human health and ecosystems is unknown also in consideration to the biopersistance of some of them. The consumer products containing micro and nanosized particles, due to wear or cracking for aging, can release NPs in the environment. At the end of the life cycle they can be disposed of , often by

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incineration. The combustive procedure can release micro and nanosized particles that lead to the formation of a diffuse pollution that involves air, water and soil. In fact the following fall-out can contaminate also soil and superficial and deep waters. Cerium is one of the rare chemicals that have been inserted as raw matter in the productive sector to be a part of the supply of consumer products. It can be found in equipment such as colours televisions, fluorescent lamps, energy saving lamps and glasses. Cerium oxide is also used in emission control systems in gasoline engines and as a diesel fuel-born catalyst to reduce particulate matter emissions. For this reason OECD has insert the CeO_2 between the 13 priority listed representative manufactured nanomaterials for immediate testing [ENV/JM/MONO(2008)13/REV].

The nanoparticles can remain in the environment for long periods and can be potentially toxic to the aquatic life (Oberdörster, 2004; Velzeboer et al., 2004) and comparatively studies have been conducted using terrestrial species (soil invertebrates, soil microorganisms, or plant) (Cristian et al., 2008; Klaine et al., 2008).

Soil is a complex aggregate of inorganic, organic and biological material. The soil aggregate have sizes ranging a larger (rocks, gravel, plant roots and debris), intermediate (organic matter, microorganisms and mineral grains) to nano scale (oxides, clays). These are often heterogeneous and coated by other minerals and organic matter. The soil is porous and hydrated and the leaching of water along the soil profile transports both nanoparticles, dissolved organic matter and inorganic species (e.g. cations and anions). The physical characteristics (e.g. porosity), as well as chemical properties (e.g. pH) play an important role in biochemical processes,. The surface of NPs provide much of the chemical reactivity for both biological and abiotic process (Navrotsky, 2004). Various processes can change the nature, and concentrations of NPs, which, as a results, can change its potential to induce toxicity.

The measurement of the soil microbial biomass (SMB), which is the major living part of the soil organic matter, has been widely adopted to assess the effect of environmental and anthropogenic pressure on soil. In fact some stress condition, that needs much time to changing the physicochemical soil's properties, can be appreciate thank the shifts of soil organisms populations or soil biotic activity (Powson et al. 1987; Pankhurst et al 1995; Giller et al. 1998).

Ecophysiological indices (metabolic quotients) are generated by basing physiological performances (respiration, growth/death, carbon uptake) on the total microbial biomass per time unit. The ratio of biomassC to soil organic C (qMic) reflects the contribution of soil microbial biomass to soil organic carbon (Anderson and Domsch, 1993). The qCO_2 (the respiration per biomass unit or metabolic quotient) has been widely used as a bioindicator of disturbance or ecosystem development. The mineralization quotient (qM) expresses the fraction of total organic carbon mineralized throughout the incubation time (Pinzari et al., 1999).

The question that must be addressed to understand the fate of NPs in the soil environment affect the ability to maintain their nominal size of nanoscale, their

original structure and their reactivity. Aim of this work was to assess the impact of the chronic exposure of CeO_2 nanoparticles (NPs) (50 to 105 nm nominal size) on soil microbial biomass and the main objective of this paper are to assess: 1) the "physical" toxicity on soil microbial biomass, due to the size (100 nm); 2) the toxicity of metals in ionic form (Ce^{3+} and Ce^{4+}) if degradation of NPs occurs.

Materials and methods

Experimental design. The NPs used in this study are Cerium Oxide (CeO_2 by NanoAmor, USA) with a purity of about a 99.9% and a spherical morphology with a diameter between 50-105 nm. They were physic-chemical characterized by means of ESEM. The soil used for the incubation experiment with CeO_2-NPs was collected at Monghidoro, Apennine of North Italy, and covered by oak forest. The soil is Epileptic Cambisol, A1 and A2 horizons were sampled, homogenised in laboratory to be carried out the experiment. The main physicochemical characteristics were: sandy clay loam texture, pH sub-acid (6.7), total organic carbon 41.96 g/kg, total nitrogen 3.20g/kg for A1 horizon; sandy clay loam texture, pH sub-acid (6.5), total organic carbon 22.29 g/kg, total nitrogen 2.11g/kg for A2 horizon. The soil analysis is carried out according to Official Methods of Soil Chemistry of Italian Agricultural Ministry (MiPAF, 2000).
100 g of both horizons, in triplicate for each thesis, are incubated in Stericup® Millipore at a constant temperature (25°C) and moisture (60% WHC). In particular, the CeO_2-NPs are added to soil in suspension, after 1 h in ultrasonic bath, at a 100 ppm concentration of Ce elements as CeO_2 and incubated in lab for short and medium times (7 and 60 days, respectively). The CeO_2-NPs concentration was chosen in order to assess the impact due to chronic progressive effects on soil of atmospheric deposition, avoiding the acute phase determined by dose $\geq$1g kg^{-1}, as shown by previous studies on nanoparticles effects on invertebrates (Coleman et al., 2010; Hu et al., 2010).

Microbial biomass Carbon (C), nitrogen (N) and ecophysiological parameters. The soil microbial biomass (SMB) was determined by the fumigation-extraction (FE) method. Three subsamples of 20 g moist soil were taken from each Stericup® Millipore and divided into two portions. One portion of 10 g moist soil were fumigated for 24 h at 25°C with ethanol-free $CHCL_3$. Following the fumigant removal, the samples were extracted with 40 ml of 0.5 M K_2SO_4 by 30 min horizontal shaking at 200 r min^{-1} and filtered (Whatman 42 filter). The non-fumigated 10 g portion was extracted similarly at the time when fumigation commenced. Organic and total (organic and inorganic) C in the extracts was measured by the Total Organic Carbon Analyser TOC-V/CPN (Shimadzu). Microbial biomass C was calculated as $E_c/k_{EC.}$ where E_c = (organic C extracted from fumigated soil)-(organic C extracted from non-fumigated soil) and k_{EC} = 0.45 (Jenkinson et al. 2004). Microbial biomass N was calculated as E_N/k_{EN} where E_N = (total N extracted from fumigated soil)-(total N extracted from non-fumigated soil) and k_{EN} =0.54 (Brookes et al., 1985; Joergensen and Muller, 1996).

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The activity of soil micro-organisms is represented by measures of soil respiration, that for each sample was measured in a closed system in accordance with the method described by Isermeyer (1952). The CO_2 evolution was performed on two replicates of each soil sample previously brought to the value of WHC by 60% and incubated at 25°C for 28th days. The measurement of CO_2 production by the soil biomass in closed environment was verified on the 1st, 3nd, 7th, 10th, 14th, 21st and 28th days for back titration to obtain constant values for each group of samples (soil respiration). The CO_2 evolved during the 10^{th} days of incubation was used as the basal respiration value.
Tree ecophysiological indexes were calculated as follows:

- $qCO_2 = (\mu g\ C\text{-}CO_{2\ basal}\ h^{-1} \times \mu g\ SMB\text{-}C^{-1}$ (Dilly and Munch, 1998)
- qMic (C_{mic}:C_{org}) = µg of SMB-C µg total organic C^{-1} (Andersen and Domsch, 1989).
- $qM = \mu g\ C\text{-}CO_{2\ cumulative}$ µg total organic C^{-1} (Pinzari et al., 1999)

Determination of microbial biomass $CHCl_3$-lable materials. The sub-samples of 20g moist soil were taken from the Stericup® Millipore and divided in two portions. One portion of 10g moist soil was fumigated for 24 h at 25°C with ethanol-free $CHCL_3$. Following the fumigant removal, the samples were extracted with 50 ml 1 M NH_4NO_3 by 60 min horizontal shaking at 200 r min^{-1} and filtered (Whatman 42 filter). The non-fumigated 10 g portion was extracted similarly at the time fumigation commenced. The laboratory materials were washed with 1 M HCl and ultrapure water (Millipore). After filtration, the extracts were acidified with HNO_3 ultrapure (Merk, Germany) (1:10 v:v ratio) and stored at 4°C. The trace elements determination in fumigated and non-fumigated extracts were carried out by Inductively Coupled Plasma with optical detection (ICP-OES, Spectro Arcos Ametek). $CHCl_3$-lable metals were calculated as ($metal_i$ extracted from fumigated soil) minus ($metal_i$ extracted by non-fumigated soil). No conversion values were applied according to Khan et al.,(2009). The metals investigated here were Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn.

Ce and metals EDTA-availability and partitioning coefficient (Kp). The degree of availability of metals in the soil samples were texted by using ethylenediaminetetraacetic acid (EDTA) extractants (Trierweiler and Lindsay, 1969) ,with an extractant to soil ratio of 10.

Water extraction (partition coefficient). The soil samples of control and CeO_2-NPs treated was extracted with deionized water (Millipore- Milli-Q) for 16 h with a ratio of 1:10 soil:solution. The extract was obtained by centrifugation for 15 minutes at 1200 x g and then filtered through 0.45 filter (Millipore). The total concentration of elements was determined by ICP-OES. Calibrations were performed by the standard solution of Bureau of Collection Recovery (BCR-909). The extraction was performed in triplicate. According to Blaser et al. (2000) an equilibrium between the soil and the aqueous solution was reached by end of the extraction has assumed. A coefficient of partition (kp) can be calculated which

relates the extractable fraction to the total concentration in soil matrix. The soil total concentration was determined after mineralization in aqua regia (2 ml HNO_3 plus 6 ml HCl) in microwave digestion system (Milestone, Start D 1200) by ICP-OES. The metals investigated were Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn.
According to the formula:

$$Kp = [metal]_{soil\ fine\ earth} / [metal]_{water\ extract} \quad [1]$$

where: Kp solid/water partition coefficient (l kg^{-1}); $[metal]_{soil\ fine\ earth}$= total metal concentration in soil (mmoli kg^{-1}); and $[metal]_{water\ extract}$= total metal concentration in water extracted (mmol l^{-1}).

Environmental scanning electron microscopy (ESEM). The soil samples were placed on an adhesive carbon disc, on an aluminium stub, and inserted in the chamber of the ESEM. This microscope was coupled to energy dispersive system (EDS) to characterize the morphology of the NPs, bulk and the surrounding soil. No coating of an electro-conductive layer on the sample surface was carried out, since the instrument used allows the observation of the actual morphology of organic and inorganic matter without any treatment, contamination or alteration.

Results

Microbial biomass Carbon (C), nitrogen (N) and ecophysiological parameters. The two horizons show a different content of microbial biomass C and N decreasing from A1 to A2 (Table 1).

Table 1 - *Soil microbial biomass C and N and organic C and total N extracted in K_2SO_4. The mean obtained by the three different incubation and standard deviation were reported.*

Time		Horizons	C_{bio}	N_{bio}	WEOC	WEN
			µg g^{-1}			
7 days	Control	A1	1108±76	103±12	243±10	134±5
		A2	400±43	59±25	108±16	53±5
	CeO_2-NPs	A1	772±59	57±3	250±7	145±2
		A2	355±45	38±10	119±10	45±5
60 days	Control	A1	912±49	26±21	197±35	139±5
		A2	368±161	43±22	82 ±10	56 ±19
	CeO_2-NPs	A1	684±20	62±22	231±14	131±11
		A2	295±45	37±1	84±23	47±25

During the incubation time there was a decrease of microbial biomass C and N in both treated and no-treated soils that means that the CeO_2-NPs treatment contributes in a high decrease of biomass (Figure 1). The difference in both microbial biomass C and N and extractable pools between the control test and CeO_2-NPs incubation is more noticeable in a short time (7 days of incubation) when the soil is more rich in organic matter (Figure 1). The presence of CeO_2 induces an increase of extractable C (WEOC) of soil.
The ecophysiological parameters of soil microbial biomass activity are shown in Table 2. CeO_2-NPs treatment increases the basal respiration that is higher in the

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poorer of C soil (A2 horizons) than A1 horizons and mostly in the first days of incubation (7 than 60 days).

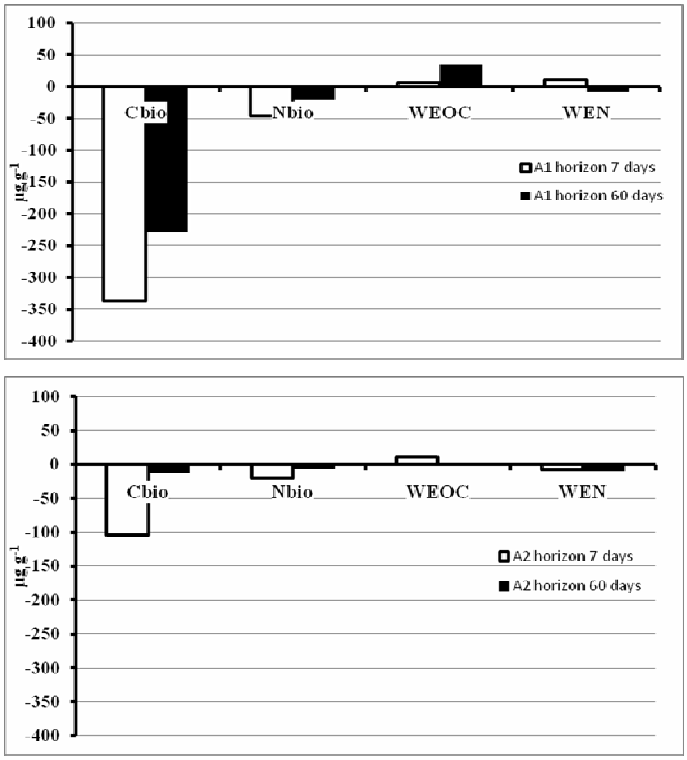

Figure 1

Difference (Δ C microbial biomass, expressed as µg g-1) between the Ce-NPs incubation and soil control at 7 (white) and 60 days (black) in A1 and A2 horizons.

The difference vanishes when the incubation time increases (Table 2) even if the difference of biomass activity in A2 horizon is always higher than A1 horizon.

Table 2 - *Some parameters of activity of soil microbial biomass are shown: Cm: C-CO_2 cumulate after 28 days fo incubation (µg C-CO_2 g^{-1}dry soil); qCO_2 is a ratio of C-CO_2 ratio (µg C-CO_2 h^{-1}) and soil microbial biomass C (µg Cmic g^{-1}); qMic is a Cmic and Corg ratio; qM is a C-CO_2 cumulate (.µg C-CO_2 g^{-1}) and Corg (mg C g^{-1} soil) ratio.*

Time		Horizons	**C_m** µg C-CO_2 g^{-1}ds	***q*CO_2** µg C-CO_2 g^{-1} h^{-1}	***q*Mic** %	***q*M** µg C-CO_2 g^{-1} C_{org}
7 days	**Control**	A1	901±87	0.89±0.01	2.3	19.0
		A2	579 ± 39	1.52±0.19	1.8	26.0
	CeO_2-NPs	A1	948±116	1.38±0.19	1.6	19.1
		A2	636±21	2.37±1.08	1.5	27.1
60 days	**Control**	A1	393±21	0.39±0.02	2.0	8.7
		A2	222±28	0.64±0.31	1.8	10.8
	CeO_2-NPs	A1	239±27	0.60±0.11	1.3	7.8
		A2	395±63	0.65±0.31	1.3	10.6

The relationship between the cumulative C-CO_2, emitted during baseline respiration, and organic C (qM) did not show differences between the thesis, while

as expected, it shows a difference depending on the incubation time. The decrease in microbial biomass C is underlined by a low microbial quotient (qMic) in the presence of nanoparticles, while the metabolic quotient (qCO_2) increases after 7 days. At 60 days of incubation, the ratio did not differ between the control and incubated soil samples.

Bioavailability of Ce and heavy metals in soil. The values of metals total concentrations in incubated soils are presented in Table 3. The background values of Ce element determined in both horizons of soil control (Ce of geogenic origin) is very similar and extraction with aqua regia is not able to extract quantitatively CeO_2-NPs added to the soil. The total values of Ce which are derived from NPs added to the soil plus geogenic-Ce, differ according to the soil organic C content (e.g. Ce total values are higher in A1 than A2 horizons).

Table 3 - *Total elements (expressed as mg kg^{-1}) in A1 and A2 horizons (the values reported are the means of three replicas of both times of incubation)*

		Cd	Ce	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Control	**A1**	0.24	46.0	12.7	44.3	35.6	37715	589	32.6	8.88	87.6
	SD	*0.00*	*2.06*	*1.62*	*6.5*	*0.66*	*264*	*35.4*	*0.83*	*5.49*	*1.61*
	A2	0.23	52.0	10.8	91.4	31.9	31060	489	27.4	3.84	77.4
	SD	*0.00*	*2.79*	*0.74*	*4.2*	*0.95*	*730*	*20.5*	*1.16*	*3.16*	*3.08*
CeO_2-NPs	**A1**	0.24	90.2	12.1	44.1	35.0	37416	564	32.1	6.61	86.8
	SD	*0.00*	*4.53*	*0.37*	*3.8*	*0.91*	*440*	*19.3*	*0.57*	*3.66*	*1.43*
	A2	0.22	56.1	10.8	38.4	31.6	36350	475	27.4	2.72	78.5
	SD	*0.00*	*19.74*	*0.54*	*3.5*	*1.37*	*182*	*29.5*	*0.99*	*3.26*	*2.55*

The values of concentrations of Ce and other metals expressed as $CHCl_3$-labile forms are shown in Table 4. The incubation with CeO_2 does not change the composition in the extract $CHCl_3$-labile. The Ce-$CHCl_3$-labile form is not extracted by control soil while in both soil horizons incubated with CeO_2 after 60 days of incubation it is found Ce after fumigation (89.6 and 41.9 µg Ce kg^{-1} in A1 and A2 horizons, respectively), while does not change the metals composition in the extract $CHCl_3$-labile. During the incubation time the metals $CHCl_3$-labile form increase both control and incubated soil, mainly in the C rich A1 horizons.

Table 4 - *$CHCl_3$-lable elements concentrations (µg kg^{-1}). The Cd values are less than detection limit (DL = 0.0-3.0 ppm) and they are not reported.*

Time			Ce	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
7 days	**Control**	**A1**	nd	6.3	nd	6.3	181.3	8725	40.6	6.3	209
		SD	*nd*	*12.5*	*33.3*	*45.8*	*12.5*	*683*	*12.5*	*nd*	*nd*
		A2	0.23	10.8	91.4	31.9	31060	489	27.4	3.84	77.4
		SD	*0.00*	*0.74*	*4.2*	*0.95*	*730*	*20.5*	*1.16*	*3.16*	*3.08*
60 days	**CeO_2-NPs**	**A1**	0.24	12.1	44.1	35.0	37416	564	32.1	6.61	86.8
		SD	*0.00*	*0.37*	*3.8*	*0.91*	*440*	*19.3*	*0.57*	*3.66*	*1.43*
		A2	0.22	10.8	38.4	31.6	18350	475	27.4	2.72	78.5
		SD	*0.00*	*0.54*	*3.5*	*1.37*	*182*	*29.5*	*0.99*	*3.26*	*2.55*

Metal concentrations extracted by EDTA show no difference between control and CeO_2-NPs treatment, and, also in this case, the increase in metals extractability is

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found after 60-day incubation (Table 5). After 60 days of incubations, the EDTA-Ce concentration of control and NPs treated soils are comparable (8.6 and 7.7 mg Ce kg^{-1} of control soil vs. 5.0 of CeO_2-treatment for A1 and A2 horizons, respectively). The geogenic and engineered origin Ce elements are not very soluble and bioavailable.

Table 5 - *EDTA-extracted (expressed mg kg^{-1})by soil control and CeO_2-NPs treatments. The Cd values are less than detection limit (DL =0.0-1.2 ppm) and they are not reported.*

Time			Ce	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
7 days	**Control**	**A1**	6.5	4.4	2.5	8.2	129.2	185.0	5.0	7.9	5.1
		SD	*0.1*	*0.0*	*0.0*	*0.2*	*1.3*	*1.8*	*0.0*	*0.4*	*0.4*
		A2	6.6	4.4	2.5	9.2	133.8	190.5	5.0	8.1	7.7
		SD	*0.1*	*0.1*	*0.0*	*1.3*	*1.8*	*3.8*	*0.1*	*0.7*	*4.3*
	CeO_2-NPs	**A1**	6.6	4.4	2.6	8.3	131.7	187.7	4.9	7.5	5.2
		SD	*0.1*	*0.1*	*0.0*	*0.3*	*3.8*	*4.2*	*0.1*	*0.2*	*0.2*
		A2	6.5	4.4	2.5	8.1	124.5	183.2	5.0	7.6	5.4
		SD	*0.1*	*0.0*	*0.0*	*0.1*	*5.8*	*3.3*	*0.0*	*0.1*	*0.4*
60 days	**Control**	**A1**	8.6	6.0	3.2	10.7	283.8	303.1	7.5	15.6	11.8
		SD	*0.1*	*0.0*	*0.0*	*0.2*	*1.3*	*1.8*	*0.0*	*0.4*	*0.4*
		A2	7.7	5.8	3.2	10.3	272.1	257.7	7.2	13.2	11.5
		SD	*0.3*	*0.1*	*0.0*	*0.2*	*1.0*	*21.2*	*0.1*	*1.7*	*0.1*
	CeO_2-NPs	**A1**	5.0	4.2	2.3	8.9	153.8	266.6	6.5	14.0	9.3
		SD	*0.1*	*0.1*	*0.0*	*1.3*	*1.8*	*3.8*	*0.1*	*0.7*	*4.3*
		A2	5.0	4.3	2.4	9.2	158.0	273.9	6.6	14.4	10.2
		SD	*0.0*	*0.1*	*0.0*	*0.1*	*2.9*	*6.2*	*0.1*	*0.4*	*0.3*

This result is highlighted also in terms of partitioning coefficient (Kp). The increase of Kp for both NPs-treated and control soil indicates a decreasing solubility. The partitioning coefficient, calculated for other elements in the soils, shows that the treatment with nanoparticles did not change the metals bioavailability (Table 6).

Table 6 - *Partition Coefficient Kp (l kg^{-1} *10^3) between the concentration of total elements in aqua regia and these of H_2O-extracted after 16 h shaking.*

Time			Cd	Ce	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
7 days	**Control**	**A1**	0.2	2.0	0.7	4.8	0.3	19.5	3.1	0.7	0.1	0.5
		A2	0.2	0.6	28.8	61.3	5.7	29.0	85.8	7.7	0.1	111.5
	CeO_2-NPs	**A1**	0.1	0.7	1.3	0.6	0.2	0.9	4.2	0.3	0.3	0.3
		A2	0.2	6.9	30.6	59.6	5.1	28.6	71.5	7.6	0.1	90.6
60 days	**Control**	**A1**	0.1	0.6	1.0	0.7	0.2	132.0	5.2	0.3	0.6	0.3
		A2	0.0	1.1	1.6	14.3	4.1	129.7	108.7	9.7	0.2	16.6
	CeO_2-NPs	**A1**	0.2	3.8	1.6	7.0	0.3	23.0	5.3	0.7	0.3	0.4
		A2	0.0	1.1	1.6	3.6	3.9	45.5	84.0	9.1	0.1	28.2

Discussion

The presence of nanoparticles in the soil can be expressed by two different types of pollution risk: the first is due to a size of NPs and it can be called "physical" while the second is due to the bioavailability of metals in ionic form (e.g. CeO_2 vs. $Ce^{+3/+4}$). The pollution risk comes from the physical size of the NPs (1-100 nm), their

distribution in porosity of soil and their ability to interact between itself, forming of cluster, and other colloids of soil (e.g. clay, oxides and humic substances) (Fang et al., 2009). Natural colloids in soil are involved in the adsorption, transport and transformation of heavy metals, organic pollutants and nutrients (Tang et al., 2009) and the transport of colloids – and therefore also NPs- in porous of soil play an important role in potentially toxicity of NPs in soil.

Soil properties such as type and size of colloids, surface area, charge behaviour, type minerals and organic matter will greatly affect the aggregation and transport of nanoparticles (Kretzschmar et al., 1997). The mobility is influenced by the Brownian diffusion due to their small size (< 350 nm) (Guzman et al., 2006) and the NPs at first are very mobile in soils. It is not yet known how the nanoparticles can be distributed according to different size pores of soils and whether single or clustered NPs are leached along the soil profiles. The redox chemistry of CeO_2-NPs, specifically the capability to cycle between the +3 and +4 oxidation states, may contribute to this unique reactivity in biological systems. The chemistry of CeO_2 in biological system is not well understood and there are many potential interactions and reactions in which the NPs can participate. A number of transition metal and lanthanide series, metal complexes are active in hydrolysis and phosphate ester bounds (Rawlings et al., 2003; Branum and Que, 1999). The phosphate ester bound is crucial for regulation of protein activity for every transfer molecules and for the stability of DNA and RNA. Ce(IV) and Ce(III) complex tend to exhibit high catalytic reactivity in this reaction (Katada et al., 2008). Ce oxides powder (1-10 μm) can dephosphorylate phosphopeptides with the high catalytic activity attributed to the multi nuclear metal complex (Tan et al., 2008). The interaction with the living part of soil (e.g. microbial biomass) can be transferred with Ce from oxides still not altered (nanoparticles) and with Ce element derived from weathering of oxides.

Ecophysiological parameters related to soil microbial biomass indicate a state of stress in the presence of CeO_2-NPs in soil. The decrease of microbial biomass C can be interpreted as a direct effect of the Ce ionic and/or CeO_2-NPs on microbial biomass or NPs engineered in soil interfere on the availability of the substrate in the conversion of microbial biomass. In fact, the NPs can increase the substrate availability increasing the dispersion of soil aggregate or the dissolution or the hydrolysis of organic matter. Many authors have shown a direct response to the change of some parameters (e.g. microbial biomass C and N, basal respiration and biomass specific respiration) in soil pollution by heavy metals (Brookes and McGrath, 1984; Brookes et al., 1986; Brookes et al., 1995; Giller et al., 1998; Nies, 1999; Vasquez-Murieta et al., 2006; Leita et al., 1995), but is well known that the ecophysiological parameters of the microbial biomass can also be a sensitive indicator of physical stress (Dilly and Munch, 1998).

The difference in C-CO_2 evolution rates between uncontaminated and NPs-contaminated soils is found in both soil horizons and Tayler (1981) reported that the decrease of soil respiration was "probably" a common feature of heavy metals pollutions of soils. The qM, or the potential C mineralization activity (measured

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under controlled conditions of temperature and humidity) did not shown significant changes meaning that NPs addition did affect the capacity of the soil to store carbon. The only consequence of this of mineralization process is an increase of C labile value (WEOC) that rises with incubation time. The specific respiration of biomass (qCO_2) increases with contamination while the qMic (microbial biomass C : total organic C ratio) decreases respect to uncontaminated soil and incubation time. The biomassC to organic C ratio indicates the substrate availability to the soil microflora or, in reverse, the fraction of recalcitrant organic matter in the soil; in fact this ratio declines as the concentration of available organic matter decreases (Brookes, 1995). The response of microbial biomass in soil polluted with NPs is very similar to that found from other authors in soil polluted by heavy metals (Brookes et al., 1995). The availability of metal in soil shows a direct relation with the decrease of microbial biomass C and its activity. The evaluation of potential risks and toxicity of metals in soil requires an assessment of the proportion of the total metals that are in a mobile and possibly bioavailable form. Extraction with chelating agents (EDTA), neutral salts (NH_4NO_3) shows no increase of Ce available form incubated with NPs compared to soil control. Conversely the NPs in soil do not change the availability of other metals in soil. The increase is bound only at the C cycle of soil.

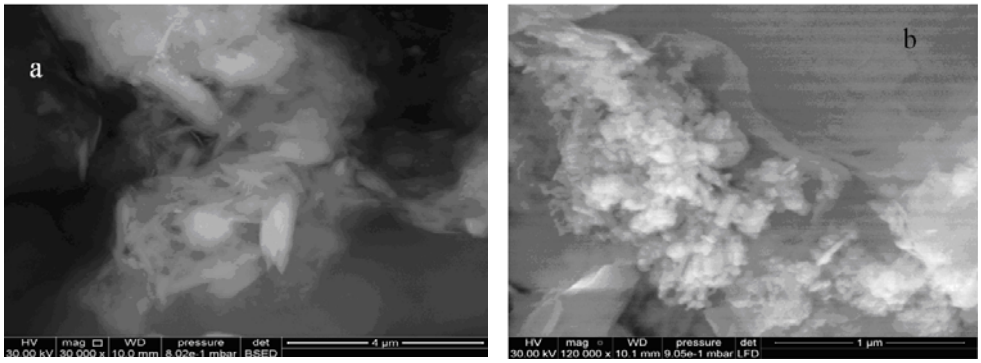
The determination of the bioavailable form can be done using a relatively simple portioning of the total metal burden between the fraction bound to soil solids and the part that is dissolved in the soil solution. The equilibrium partitioning is generally used to predict the partition of a substance between two compartments of a given medium, which is solid phase constituted by sediments or soils, and pores water (Haye et al., 2007). The portioning coefficient Kp, defined as the partition coefficient of a substance between the solid phase and pores water (expressed as the ratio concentration in soil (mg kg^{-1}) to the concentration in pores water (mg l^{-1}) in l kg^{-1}) is a key parameter to assess the behaviour and toxicity of substances (Haye et al., 2007). The high values of Kp for both treated and no-treated CeO_2-NPs samples indicate a low solubility. The dissolved metal pool also reflects the soil metal fraction that could potentially be leached from the soil and contaminate groundwater and surface waters.

The very short incubation time indicates an insolubility from of Ce oxide added into soil as nanoparticles. The partitioning coefficient of some metals found in both horizons polluted by CeO_2-NPs has a behaviour similar that observed by other authors (Sauvé et al., 2000) and their changes during the incubation time can be attributed mostly to changes in physical-chemical characteristics of soil. Mainly the incubation time and established conditions play an important role on the liquid/soil ratio (Kp) of other metals. In this work the Ce and other metals are available in the soil richer in organic matter (Sauvé et al., 2000).

CeO_2 NPs were clearly detected in soil and recognized by ESEM - EDS characterization. The NPs chemical composition appears unaltered, while the size can be modified by NPs aggregation and clustering (Figure 2). Natural organic

matter (NOM) is expected to have important influences on physicochemical reactions.

Figure 2 - *SEM images of CeO2 NPs in different aggregated in soils.*



The exact role of NOM in colloid stabilization or destabilization would primarily depend on chemical properties such as size, charge and rigidity of various functional groups (Wilkison et al., 1997). Fulvic acid was shown to stabilize colloidal solutions (Wilkison et al., 1997); humic acid was also frequently reported for effectively stabilizing nanoparticles suspensions by steric repulsion effect (Chen and Elimelech, 2007; Hyung et al., 2007).

Ecophysiological parameters of microbial biomass show a state of metabolic stress in the first seven days of incubation, but as seen from the ESEM images the NPs remain in contact with the microbial biomass in the pores of soil. CeO_2 particles were adsorbed onto the cell of Escherichia coli (Thill et al., 2006), NPs that damage bacterial cell walls have been found to be internalized, whereas those without this activity were not taken up (Stoimenov et al., 2002). There is an increase of C/N ratio of soil microbial biomass from 6.8 and 8.6 to 10.7 and 11.1 for A1 and A2 horizons respectively in control and treated soil samples. Higher values are found in NPs polluted soil highlighting a microbial population change. The bacteria have a C/N ratio of 6.5, which can rise to 8.2-8.3 in the grassland and forest soils, which is similar to the values found in A1 and A2 horizons of Cambisol used in this study, while the increase during the incubation with NPs suggest a predominantly fungal population (Cleveland and Lipzin, 2007).

The soil microbial biomass is affected by NPs and the Ce concentrations in $CHCl_3$-labile pool in soil treated with CeO_2 must be taken into account. For the concentration of the other metals, the content of $CHCl_3$-labile manganese was high indicating effects of the metal composition in soil (Khan et al., 2009). Co, Cr, Ni content was mostly due to geogenic background and it was not influenced by NPs addition into soil.

It is not yet known the long-term behaviour of aggregates of engineered NPs into soil; in fact they can be stabilized by organic matter or, like the natural colloids,

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can be altered by physical-chemical and biochemical processes, changing the behaviour and fate of NPs in soil

Conclusions

The presence of CeO2 NPs was clearly detected in soil and recognized by ESEM morphological observations coupled with EDS characterization. The NPs chemical composition appears unaltered, while the size can be modified by NPs aggregation and clustering.
Our result of the biological assays shows a stressful effect of NPs on soil microbial biomass at 7-day, that stress vanishes at 60-day. The disappearance of the negative effect of nanoparticles on the microbial biomass at 60-day is hypotized to: a change in soil microbial biomass (fungal vs bacteria) and an aggregation and clustering of NPs with the NOM (less surface to volume ratio). We have shown that there isn't a risk of bioavailability of metals in ionic form in short term, but we don't know the long-term behaviour of this compound. In fact, if soil microbial biomass changes, can also change the process of interactions between NPs and organic products (e.g. enzymes, organic acids, dissolved organic matter) favouring to a release of metal ions from the cluster of NPs

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References

ANDERSON T.H., DOMSCH K.H. (1993) The metabolic quotient for CO_2 (qCO_2) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils. Soil Biology & Biochemistry 25:393–395.
BEKYAROVA E., FORNASIERO P., KAŠPAR J., GRAZIANI M. (1998) CO oxidation on $Pd/CeO_2{\pm}ZrO_2$ catalysts. Catalysis Today 45:179-183.
BRANUM M.E., QUE JR. L. (1999) Double-strand DNA hydrolysis by dialnthanide complexes. Journal of Biological Inorganic Chemistry 4:593-600.
BROOKES P.C., LANDMAN A., PRUDEN G., JENKINSON D.S. (1985) Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method for measuring microbial biomass nitrogen in soil. Soil Biology & Biochemistry 17:837-842.
BROOKES PC. (1995) The use of microbial parameters in monitoring soil pollution by heavy metals. Biology and Fertility of Soils 19:269-279.
CLEVELAND C.C., LIPTZIN D. (2007) C:N:P stoichiometry in soil: is there a "redfield ratio" for the microbial biomass. Biogeochemistry 85:235-252.
COLEMAN J.G., JOHNSON D.R., STANLEY J.K., BEDNAR A.J., WEISS C.A., BOYD, R.E., STEEVENS J.A. (2010) Assessing the fate and effects of nano aluminum oxide in the terrestrial earthworm, Eisenia fetida. Environmental Toxicology & Chemistry 29:1575–80.
CHRISTIAN P., VON DER KAMMER F., BAALOUSHA M., HOFMANN T. (2008)

Nanoparticles: Structure, properties, preparation and behavior in environmental media. Ecotoxicology 17:326–343.
DILLY O., MUNCH J.C. (1998) Ratios between estimates of microbial biomass content and microbial activity in soils. Biology and fertility of Soils, 27:374-379.
FANG J., SHIAN X., WEN B., LIN J., OWENSG. (2009) Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil column. Environmental pollution 157:1101-1109.
GILLER K.E., WITTER E., McGRATH S.P. (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology & Biochemistry 30:1389–1414.
HAY S., SLAVEYKOVA V.I., PAYET J. (2007) Terrestrial ecotoxicity and effect factors of metals in life cycle assessment (LCA). Chemosphere 68:1489-1496.
HU C.W., LI M., CUI Y.B., LI D.S., CHEN J., YANG L.Y. (2010) Toxicological effects of TiO_2 and ZnO nanoparticles in soil on earthworm Eisenia fetida. Soil Biology & Biochemistry 42:586–91.
LEITA L., DE NOBILI M., MUHLBACHOVA G., MONDINI C., MARCHIOL L., ZERBI G., (1995) Bioavailability and effects of heavy metals on soil microbial biomass serviva during laboratory incubation. Biology and Fertility of Soils 19:103-108.
JENKINSON D.S., BROOKES P.C., POWLSON D.S. (2004) Measuring soil microbial biomass. Soil Biology & Biochemistry 36:5–7.
JOERGENSEN R.G., MUELLER T. (1996) The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{EN} value. Soil Biology & Biochemistry 28:33-37.
KATADA H., SEINO H., MIZOBE Y., SUMAOKA J., KOMIYAMA M. (2008) Crystal structure of Ce (IV)/dipicolinate complex as a catalyst for DNA hydrolysis. Journal of Biological Inorganic Chemistry 13:249-255.
KHAN K.S., HEINZE S., JOERGENSEN R.G. (2009) Simultaneous measurements of S. macronutrients. and heavy metals in the soil microbial biomass with $CHCl_3$ fumigation and NH_4NO_3 extraction. Soil Biology & Biochemistry 41:309-314.
KLAINE S.J., ALVAREZ P.J.J., BATLEY G.E., FERNANDES T.F., HANDY R.D., LYON D.Y., MAHENDRA S., MCLAUGHLIN M.J., LEAD J.R. (2008). Nanomaterials in the environment: Behavior, fate, bioavailability and effects. Environonmental Toxicology Chemestry 27:1825–1851.
KRETZSCHMAR R., BARMETTLER K., GROLIMUND D., YAN Y., BORKOVEC M., STICHER H. (1997) Experimental determination of colloid deposition rates and collision efficiencies in natural pour media. Water Resources Research 33:1129-1137.
KUZMAN K.A.D., FINNEGAN M.P., BANFIELD J.F. (2006) Influence of surface potential on aggregation and transport of titania nanoparticles. Environmental Science & Technology 40:7688-7693.
MCDONALD J.W., GHIO A.J., SHEEHAN C.E., BERNHARDT P.F., ROGGLI V.L. (1995) Rare earth (cerium oxide) pneumoconiosis: analytical scanning electron microscopy and literature review. Modern Pathology 8:859–865.
NAVROTSKY A. (2004) Encyclopedia of Nanoscience and Nanotechnology. Marcel Dekker, Inc.
NAKAGAWA K., MURATA Y., KISHIDA M., ADACHI M., HIRO M., SUSA K. (2007) Formation and reaction activity of CeO_2 nanoparticles of cubic structure and various shaped CeO_2-TiO_2 composite nanostructures. Materials Chemistry and Physics104:30–39.
PAIRON J.C., ROOS F., IWATSUBO Y., JANSON X., BILLON-GALLAND M.A., BIGNONO J., BROCHARD P. (1994) Lung retention of cerium in humans. Occupational and Environmental Medicine 51:195–199.
DOI: 10.6092/issn.2281-4485/3829

PARK B., MARTIN P., HARRIS C., GUEST R., WHITTINGHAM A., JENKINSON P., HANDLEY J. (2007) Initial in vitro screening approach to investigate the potential health and environmental hazards of EnviroxTM – a nanoparticlulate cerium oxide diesel fuel additive. Particle and Fibre Toxicology 4, 12.

PANKHURST C.E., HAWKE B.G., MCDONALD H.J., KIRKBY C.A., BUCKERFIELD J.C., MICHELSEN P., O'BRIEN K.A., GUPTA V.V.S.R, DOUBE B.M. (1995) Evaluation of soil biological properties as potential bioindicators of soil health. Australian Journal of Experimental Agriculture 35:1015–1028.

PINZARI F., TRINCHERA A., BENEDETTI A., SEQUI, P. (1999) Use of biochemical indices in the Mediterranean environment: comparison among soil and different forest vegetation. Journal od Microbiological Method 36:21-28.

POWLSON D.S., BROOKES P.C., CHRISTENSEN B.T. (1987) Measurement of soil microbial biomass provides an early indication of changes in total soil organic matter due to straw incorporation. Soil Biology & Biochemistry 19:159–164.

QUIK J.T.K., LYNCH I., VAN HOECKE K., MIERMANS C.J.H., DE SCHAMPHELAERE K.A.C., JANSSEN C.R., DAWSON K.A., COHEN STUART M.A., VAN DE MEENT D. (2010) Effect of natural organic matter on cerium dioxide nanoparticles settling in model fresh water. Chemosphere 81:711-715.

RAWLINGS J., CLELAND W.W., HENGGE A.C. (2003) Metal ion catalyzed hydrolysis of ethyl p-nitrophenyl phosphate. Journal Inorganic Biochemistry 93:61- 65.

SATO T., KATAKURA T., YIN S., FUJIMOTO T., YABE S. (2004) Synthesis and UV-shielding properties of calcia-dopedCeO_2 nanoparticles coated with amorphous silica. Solid State Ionics 172:377–382.

SOUVÉ S., HENDERSHOT W., ALLEN H. (2000) Solid-liquid partitioning of metals in contaminated soil: dependence on pH. total metal burden. and organic matter. Environmental Science &Technology 34:1125-1131.

STOIMENOV P.K., KLINGER R.L., MARCHIN G.L., KLABUNDE K.J. (2002) Metal oxide nanoparticles as bactericidal agents. Langmuir 18:6679–6686.

TAN F., ZHANG Y., WANG J., WIE J., CAI Y., QIAN X. (2008) An efficient method for dephosphorylation of phosphopeptides by cerium oxide. Journal of Mass Spectrometry 43: 628-632.

TANG Z., WU L., LUO Y., CRISTIE P. (2009) Size fractionation and characterization of nanocolloidal particles in soils. Environmental Geochemical Health 31:1–10

THILL A., ZEYONS O., SPALLA O., CHAUVAT F., ROSE J., AUFFAN M., FLANK A.M. (2006) Cytotoxicity of CeO2 nanoparticles for *Escherichia coli*. Physico-chemical insight of the cytotoxicity mechanism. Environmental. Science. Technology 40:6151–6156.

TRIERWEILER J.F., LINDSAY W.L. (1969) EDTA-ammonium carbonate soil test for Zn. Soil Science Society of America Proceeding 33:49-54.

VAN HOECKE K., QUIK J.T.K., MANKIEWICZ-BOCZEK J., DE SCHAMPHELAERE K.A.C., ELSAESSER A., VAN DER MEEREN P., BARNES C., MCKERR G. , HOWARD C.V., VAN DE MEENT D., RYDZYŃSKI K., DAWSON K.A. , SALVATI A., LESNIAK A., LYNCH I., SILVERSMIT G., DE SAMBER B. , VINCZE L., JANSSEN C.R. (2009) Fate and effects of CeO_2 nanoparticles in aquatic ecotoxicity tests, Environmental Science and Technology 43:4537-4546.