EQA *– Environmental quality / Qualité de l'Environnement / Qualità ambientale, 8 (2012) 51-60*

TOXICOLOGICAL EFFECTS OF ENGINEERED NANOPARTICLES ON EARTHWORMS (*LUMBRICUS RUBELLUS*) IN SHORT EXPOSURE

EFFETS TOXIQUES DE LES NANOPARTICULES SUR LOMBRICO (*LUMBRICUS RUBELLUS*) DANS EXPOSITION COURT

EFFETTI TOSSICI DI NANOPARTICELLE INGEGNERIZZATE SU LOMBRICO (*LUMBRICUS RUBELLUS*) NELLA BREVE ESPOSIZIONE

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Abstract

Nanoparticles (NPs) types diffused into the environment are increasing, giving potential damages to terrestrial ecosystems. In this work we investigated the nanoparticles toxicity as pure metal and two oxides (Co, SnO_2 and CeO_2) on earthworm survival (*Lumbricus rubellus*). A concentration of 5000 mg kg^{-1} dry soil of each NP was compared to the chronic dose of 10 mg kg^{-1}. The interaction between NPs and soil microbial biomass was also studied in 7 days length incubation. No mortality was observed at the end of the experiment, but high concentration of Co was found in the 5000 kg^{-1} dry soil treated. Despite low solubility of all NPs (solid-liquid partition coefficient > 2.8 log l kg^{-1}) pure metal NPs were (Co) more soluble than the metal oxides nanoparticles (SnO_2 and CeO_2).

Keywords: *earthworms; nanoparticles; toxicity; soil*

Résumé

Les nanoparticules (NPs) sont libérés dans l'environnent dans des proportions sans cesse croissantes. Dans le présent document nous étudions la toxicité des nanoparticules sous la forme de métal pur (Co) et de deux oxydes métalliques d'étain et de cérium (SnO_2 et CeO_2) sur les vers de terre (*Lumbricus rubellus*). L'expérience implique la comparaison entre une dose chronique (10 mg kg^{-1}) et que aiguë (5000 mg kg^{-1}). Dans ce travail, nous avons pris en compte les interactions entre la biomasse microbienne du sol et les NPs. Pendant 7 jours d'incubation les vers de terre sont tous les survivants de différentes doses de NPs, bien que les doses élevées de Co-NPs ont été retrouvés dans les tissus des lombrics. Les nanoparticules de Co sont plus solubles que les autres oxydes testés dans cette expérience, même si le coefficient de partage solide-liquide a souligné une faible solubilité de nanoparticules dans le sol.

DOI: 10.6092/issn.2281-4485/3750

Mots clés: *ver de terre; les particules nano; toxicité; sol*

Riassunto

Le nanoparticelle (NPs) vengono rilasciate nell’ambiente in proporzioni sempre maggiori determinando quindi potenziali danni agli ecosistemi terrestri. In questo lavoro viene investigata la tossicità delle nanoparticelle in forma di metallo puro e di due ossidi (Co, SnO_2 e CeO_2) sui lombrichi (*Lumbricus rubellus*). L’esperimento prevede il confronto tra una dose cronica e ed un’acuta, rispettivamente 10 e 5000 mg kg^{-1}. Inoltre, sono state prese in considerazione le interazioni tra la biomassa microbica del suolo e le NPs in 7 giorni di incubazione. Nessuna mortalità è stata osservata alla fine dell'esperimento, ma alte concentrazioni di Co-NPs sono state trovate nei lombrichi. Dai risultati si evince che le nanoparticelle di Co sono più solubili rispetto agli altri ossidi testati, anche se il coefficiente di partizione solido-liquido sottolineava una bassa solubilità nel suolo di tutte le nanoparticelle.

Parole chiave: *lombrico; nano particelle; tossicità; suolo*

Introduction

The nanometal particles (NPs) exist in natural systems such soils, groundwater, streams, rivers, oceans and in atmosphere (Tsao *et al*., 2011). Nanoscale engineered material are synthesize and manipulate by man and they are used in a variety of different areas as electronic, biomedical, pharmaceutical, cosmetic, environmental, agricultural, food (Nowack and Bucheli, 2007). The forecasted huge increase in the manufacture (>58,000 tons in 2011-2012, Maynard, 2006) and use of NPs makes it likely that increasing human and environmental exposure to these materials will occur. It is unavoidable that NPs will be released into environment intentionally or accidentally. The potential risk and potential harmful effects of NPs exposure remain unclear also their toxicological impacts on environmental compartments must be clarified.

The soil seems to be the ultimate sink for a significant fraction of NPs released to the environment mainly due to addition of sewage sludge contaminated by NPs. Nanomaterials are also employees in agricultural practices as pesticides and fertilizers (Kuzma and VerHage, 2006). The response of the environment to nanoparticles can be extremely complex and diverse, depending on a variety parameters involved, which makes difficult modelling their environmental fate. In general, the knowledge about potential environmental hazards (Klaine et al, 2008) are poor.

The earthworms play an important role in terrestrial ecosystems (Edwards and Bohlen, 1996) and they are common test organisms in soil ecotoxicity studies and are used to assess bioavailability of potentially hazardous materials (e.g. heavy metals) in soil (Ma, 2005). Recent studies have used earthworms to assess the specific properties of engineered NPs with respect to both toxicity and bioavailability (El-Temsah and Joner, 2012; Hu *et al*, 2010; Unrine *et al.,* 2010 a, b; Vittori Antisari *et al.,* 2011).

Recent nano-ecotoxicological studies indicate so far that an increasing dose provides an increase in response, although this may not follow traditional mass based dose–response relationships (e.g. Oberdorster *et al.,* 2005).

In the present study we screened the toxicity of a pure metal based NPs (Co), two metal oxide based NPs (SnO_2 and CeO_2) to the earthworm *Lumbricus rubellus*. A concentration of 5000 mg kg^{-1} dry soil of each NP was compared to the chronic dose of 10mg kg^{-1} by the limit-test design.

Material and Methods

Experimental design

In order to verify bioaccumulation in earthworms from contaminated soils by NPs has already been done pilot tests carried out by mixing a technologic soil (INESE soil: sandy clay loam, pH moderately alkaline, TOC 49.50 mg kg^{-1}, TKN 3.20 mg kg^{-1}) with Co and SnO_2 CeO_2 NPs. A chronic dose of 10 mg kg^{-1} d. s. was compared to the an acute dose 5000 mg kg^{-1} d. s of each NP, NP1 and NP2 respectively. Hundred grams of technologic soil were weight in glass jars, with a volume of 500ml, and then incubated for 7 days at temperature and soil moisture controlled (i.e. 25°C and 60% WHC). Mature specimens were selected from nursery and added to each replicate samples. The earthworms were fed with piece of apple.

According to the method in "Guidelines for the testing of chemicals" (OECD, 2010) after the incubation half of the earthworms were killed immediately (freezing at temperatures below -18°C) and the other half was allowed to purge their gut overnight and then killed.

Soil analysis

Soil microbial biomass and its activity. The soil microbial biomass (SMB) was determined by the fumigation-extraction (FE) method according to Vance et al., 1987. Potassium sulphate extractable C and N in fumigated and no fumigated samples was measured by the Total Organic Carbon Analyser TOC-V/CPN (Shimadzu).

Microbial biomass C (SMB_C) was calculated as $EC/k_{EC.}$ where EC = (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 (SMB_N) was calculated as EN/k_{EN} where EN = (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). In addition, the no fumigated samples were used to assess the available pools C and N (WEOC and WEON according to Badalucco (1992). The activity of soil micro-organisms was represented by measures of soil respiration, which 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

DOI: 10.6092/issn.2281-4485/3750

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 respiration rate between the 14th and 21st day of incubation was used as the basal respiration value to calculate the metabolic quotient as follows: qCO_2= (µg $C\text{-}CO_{2basal}$ h^{-1} x µg $Cmic^{-1}$) x 10^3 (Dilly and Munch, 1998).

Nanoparticles bioavailability. The nanoparticles bioavailability was assessed as solubility in different extractants: H_2O, NH_4NO_3, EDTA, aqua regia HCl:HNO_3 (2:1 v/v). In addition, water and aqua regia extracts were used to calculate the solid-liquid partition coefficient (Kp) which relates the extractable fraction to the total concentration in soil matrix in accord to Blaser et al. (2000).

The fumigated and no fumigated samples were extracted with 50 ml 1M NH_4NO_3 by 60 min horizontal shaking at 200 r min^{-1} and filtered (Whatman 42 filter). After filtration, the extracts were acidified with HNO_3 suprapur (Merck, Germany) (1:10 v/v) and stored at 4°C. The trace elements determination in fumigated and non-fumigated extracts was carried out by Inductively Coupled Plasma with optical detection spectrometry (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 degree of availability of metals in the soil samples were texted by using ethylenediaminetetraacetic acid (EDTA) solution (Trierweiler and Lindsay, 1969), with a ratio of 1:10 w/v and NH_4NO_3 1M solution with a ratio of 1:5 w/v. The extraction for each treatment was performed in triplicate.

The control and NPs treated soil samples were extracted with deionizer water (Milli-Q) for 16 h with a ratio of 1:10 w/v. 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. 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 (Spectro Arcos Ametek). According to the following equation:

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

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

Earthworms (Lumbricus rubellus) characterization. The earthworms’ tissues and their faeces were mineralized in PTFE bombs with HNO_3 suprapur (Merck, Germany) in a microwave oven. The mineralization solution was brought at 20 ml volume with deionised water (Milli-Q) and then the samples were filtered with cellulose filters (Whatman 42). The major and trace elements were determined by ICP-OES. Purged earthworms were fixed with 2% paraformaldehyde and prepared for microscopic inspection. The NPs present inside the earthworms' tissues were identified by ESEM, equipped with EDS (Quanta FEI-250). The electron microscope analyses were performed by catching Back Side Electrons (BSE), in order to obtain information on the chemical nature of the samples, rather than their morphology.

Statistical analysis. All experiments were made in 3 replicates and the results are presented as means (± SD) using the software Excel (Microsoft).

Results and discussion

Table 1 shows the amount of soil microbial biomass C and N (SMB_C and SMB_N) and available pools C and N (WEOC and WEON) in soil polluted with both doses of different NPs, namely Co, CeO_2 and SnO_2.

Table 1 - *Amount of soil microbial biomass C and N (SMB_C and SMB_N, respectively) and K_2SO_4-extractable C and N (WEOC and WEON, respectively) for two different doses of NP (NP1 and NP2)*

	SMB_C	**SD**	**SMB_N**	**SD**	**WEOC**	**SD**	**WEON**	**SD**
				mg kg^{-1}				
NP-0	**1096**	**173**	**134**	**7**	**403**	**44**	**63**	**25**
CeO_2-NP1	**1147**	**236**	**108**	**35**	**334**	**72**	**91**	**15**
CeO_2-NP2	**788**	**35**	**56**	**18**	**530**	**23**	**110**	**16**
Co-NP1	**1037**	**64**	**107**	**21**	**386**	**2**	**95**	**2**
Co-NP2	**951**	**230**	**98**	**12**	**625**	**17**	**36**	**2**
SnO_2-NP1	**905**	**214**	**95**	**32**	**548**	**116**	**41**	**9**
SnO_2-NP2	**1036**	**53**	**123**	**2**	**462**	**8**	**94**	**5**

No significant difference of SMB_C and SMB_N was found in INESE soil incubated with earthworms at different doses of NPs, as shown in Table 1. The highest dose of Co-NP showed an increase of available C and a decrease of available N pool respect to control test. These phenomena can be attributed to interaction between microbial membrane and NPs.

The microbial membrane of bacteria is loaded and then their interaction in soil with particles that were characterized by high charge and high surface area can induce stress. The reports on the toxicity of oxides NPs on soil microbial biomass are contradictory (Dinesh *et al.*, 2012).

The basal respiration was measured to determine the metabolic quotient (qCO_2) which assesses the metabolic stress of SMB and it is shown in Figure 1.

No statistical difference in metabolic ratio qCO2 between NPs polluted and unpolluted soil was found, even if the metabolic quotient increase with highest

DOI: 10.6092/issn.2281-4485/3750

doses. This increase was probably due to the stress of microbial biomass in presence of NPs.

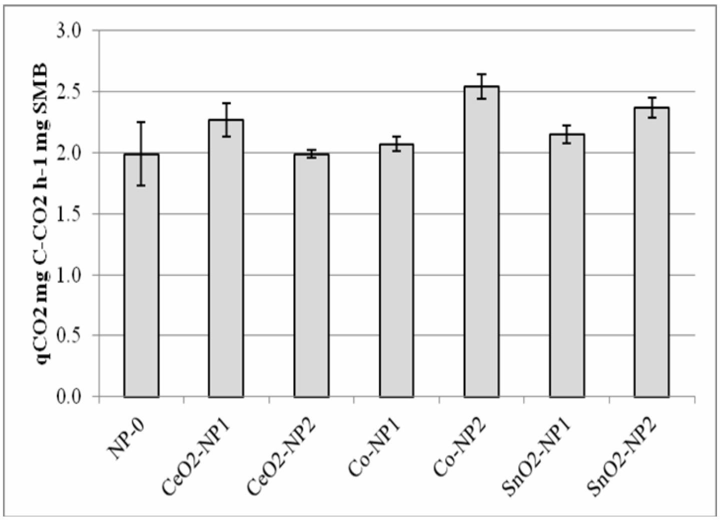


Figure 1 –

Metabolic quotient (qCO_2, mg C-CO_2 mg^{-1} SMB h^{-1}) of SMB is soil polluted with different NPs.

$CHCl_3$ labile metals were determined from fumigated and no fumigated soil samples, after extraction with NH_4NO_3 only at highest dose of NPs. Tin was not found in both unpolluted and polluted samples, Cerium was not found in untreated samples while in polluted soil 110 (± 0.05) µg Ce kg^{-1} was detected. Cobalt was detected in both control test (5 ± 0.001 µg Co kg^{-1}) and polluted soil (3304 ± 0.2 µg Co kg^{-1}). This last result highlighted a more solubility of metal NP (Co) than oxide nanoparticles (SnO_2 and CeO_2). The cobalt high-availability was confirmed by the $CHCl_3$-labile extract.

The data of availability of elements in polluted soil with different NPs are reported in Table 2.

Generally, the extraction strength follows this trend: $H_2O<NH_4NO_3<$EDTA, but tin was an exception. Indeed tin was detectable in water and not detectable in NH_4NO_3 and EDTA extracts, this could be explained by the interaction of SnO_2-NPs with some natural colloids that remain in suspension in distilled water, but not in the other extractants.

As seen, Co was more soluble than oxide nanoparticles, Co extracted in EDTA was 27% of that found in aqua regia. Aqua regia was not able to recover the nominal doses of NPs added in soil. The log Kp was higher than 2.8 and this indicates a low solubility of NPs added to the soil.

The coefficient of solid-solution partitions of metals in all polluted soils was higher than 2.8, stressing a low solubility of metal and metal oxides NPs. No toxic effect in earthworms due to the contact and ingestion of polluted soil with NPs was found. All earthworms of each thesis were survived after 7 days incubation at both “chronic” and acute doses.

Table 2 - *Available elements extracted with different strength solution (e.g. H_2O, NH_4NO_3, EDTA) and total elements determined in soil with aqua regia. The log Kp is the coefficient of partition between solid and aqueous phase calculated according Blaser et al. (2000). SD is standard deviation, while ND is not detectable.*

	H_2O	SD	NH_4NO_3	SD	EDTA	SD	AR	SD	log Kp
	$\mu g\ kg^{-1}$		$mg\ kg^{-1}$		$mg\ kg^{-1}$		$mg\ kg^{-1}$		$log\ l\ kg^{-1}$
NP-0 (Ce)	ND	-	0.3	0.01	6.9	0.1	24.3	0.9	ND
CeO_2-NP1	ND	-	0.3	0.07	7.3	0.1	29.3	1.0	ND
CeO_2-NP2	ND	-	0.3	0.03	7.4	0.2	44.7	4.1	ND
NP-0 (Co)	3.6	0.07	0.01	0.001	0.9	0.03	5.8	0.2	3.2
Co-NP1	18.3	0.02	1.2	0.002	1.7	0.15	10.2	1.6	2.7
Co-NP2	5249	501	20.2	2.2	1041	174	3792	496	2.9
NP-0 (Sn)	ND	-	ND	-	ND	-	6.5	3.4	ND
SnO_2-NP1	12.3	0.2	ND	-	ND	-	33.7	0.2	3.4
SnO_2-NP2	1972	137	ND	-	ND	-	2564	86.5	3.1

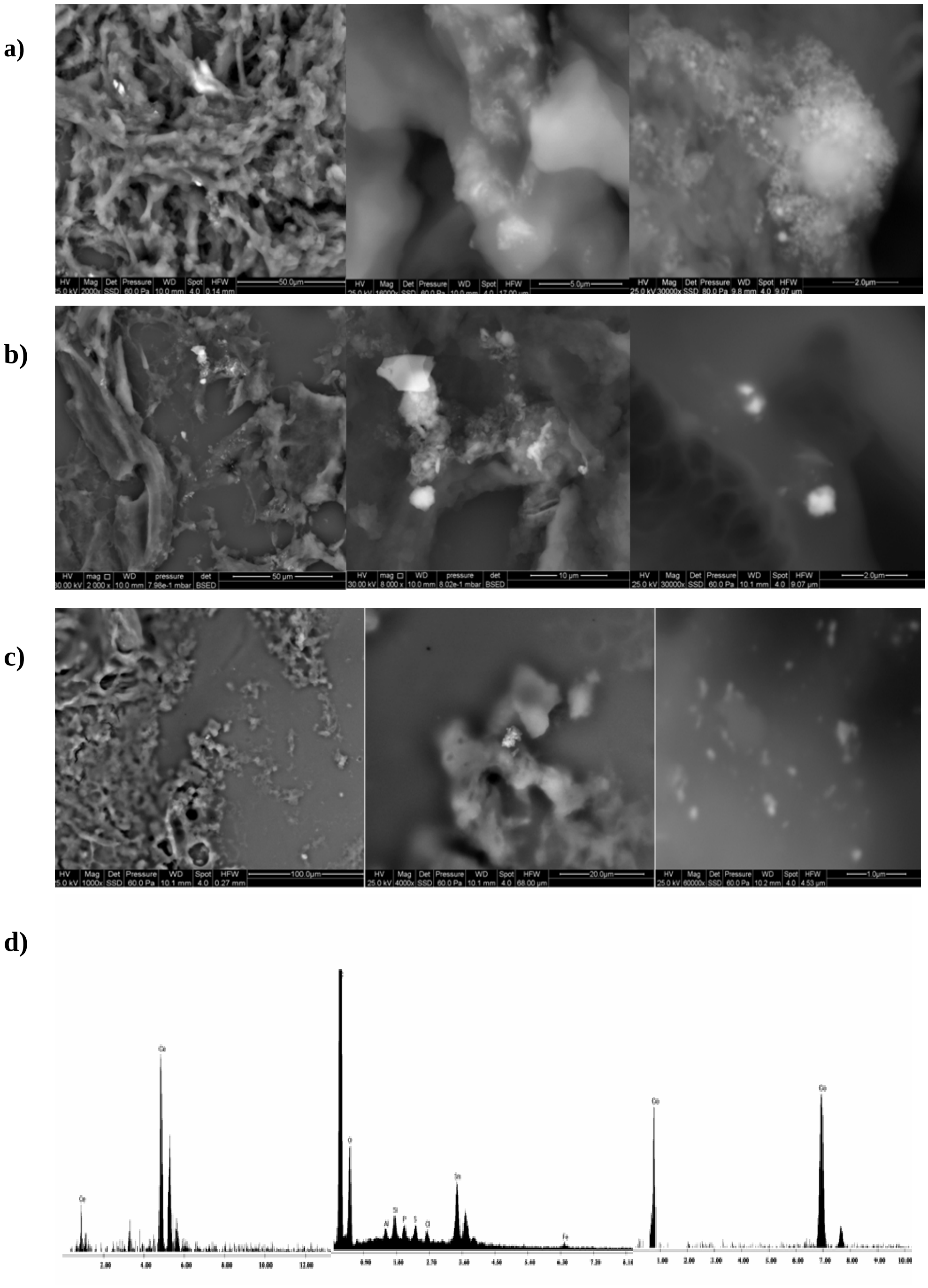
The total concentrations of elements in earthworms' tissues and faeces are shown in Table 3. The cobalt high-availability was confirmed by the high concentration found in earthworms' tissues and faeces. This last result was corroborated by those obtained from aqua regia mineralization of soil. Instead, high concentrations of Sn at acute dose were found in soil, while it was not detected in earthworms' tissues. Earthworms, purged for 24h, were placed in paraformaldehyde and then prepared for analysis by electron microscopy. The investigation and characterization by ESEM-EDS detected the NPs in purged bodies of earthworms, as clearly shown in Figure 2.

Nanoparticles		Ce	Co	Sn
			$\mu g\ g^{-1}$	
NP-0	**earthworm**	**2.3**	**7.5**	**0.8**
	faeces	**28**	**6.2**	**0.3**
CeO_2-NP1	**earthworm**	**3.6**	**-**	**-**
	faeces	**33**	**-**	**-**
CeO_2-NP2	**earthworm**	**5.3**	**-**	**-**
	faeces	**49**	**-**	**-**
Co-NP1	**earthworm**	**-**	**30.4**	**-**
	faeces	**-**	**30.3**	**-**
Co-NP2	**earthworm**	**-**	**2742**	**-**
	faeces	**-**	**2812**	**-**
SnO_2-NP1	**earthworm**	**-**	**-**	**3.9**
	faeces	**-**	**-**	**23.2**
SnO_2-NP2	**earthworm**	**-**	**-**	**2.1**
	faeces	**-**	**-**	**49.1**

Table 3

Concentrations of elements of nanoparticles in eartworms' tissues and faeces.

Figure 2 - *Electron microscope immages of NPs in earthworms tissues. In particular a) CeO_2 immages; b) SnO_2 immages; c) Co immages and then d) spectra obtained by microprobe analysis..*



Conclusion

In conclusion, these results contribute to the setting of reference baseline values of NPs in soil. Cobalt nanoparticles, due to their high-availability, seem to have the most stressful effect on soil microbial biomass as shown by the increase of available C and a decrease of available N pool. All the NPs enter into the earthworms intestine, but they have no acute toxic effect.

Further experimental investigations are needed to estimate the effect of engineered nanoparticles in long term exposure.

Acknowledgements

The research was supported by the Italian Institute of Technology of Italy: project INESE 2010-13.

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