Ecotoxicological effects of nanomaterials in terrestrial and marine environments. An integrated approach in ENEA – Portici

Is there an environmental concern in the ever increasing use of nanomaterials in many aspects of our lives? Ecotoxicologists are now facing a formidable challenge that is: trying to assess the impact of "nano" on the environment well knowing that a lot of scientific and technological information on the physics and chemistry of nanomaterials is still missing.
In this work, the ENEA Portici team researching on the field reports on evidence of toxicological effects on the environment of nano ZnO, a nanomaterial widely used in the health and fitness industry

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Effetti ecotossicologici di nanomateriali in ambiente marino e terrestre. L'approccio integrato dei ricercatori ENEA di Portici

Esiste una reale preoccupazione per l'ambiente determinata dal costante aumento dell'utilizzo dei nanomateriali in molti aspetti della nostra vita? Gli ecotossicologi stanno affrontando una sfida formidabile: tentare di valutare l'impatto del "nano" in campo ambientale consapevoli che molte informazioni scientifiche e tecnologiche sulla fisica e la chimica dei nanomateriali sono ancora insufficienti.
In questo lavoro, i ricercatori del Centro ENEA di Portici che operano in questo campo, riportano alcune evidenze di effetti ecotossicologici dell'ossido di zinco nanostrutturato, un materiale largamente utilizzato nel campo della salute e del fitness

Nanotechnology is a rapidly expanding field of research continuously producing a variety of commercial items such as cosmetics, paints, self-cleaning glasses, stain-resistant clothing and fascinating electronic appliances.
The amount of consumer products containing nanoparticles (NPs) or nanofibers is rapidly growing: from the 212 products in March 2006, to the 609 in February 2008 and up to 1,300 counted in March 2011 (Project on Emerging Nanotechnologies, 2011)[1]. In Table 1, the most relevant nanotechnological sectors and the relative number of products are reported. Due to this rapid diffusion of nano-based product, the occupational and public exposure to NPs is supposed to dramatically increase in coming years as well as concern about potential adverse effects on the environment. The risks related to their uncontrolled diffusion in the environment have been long neglected till, in 2004, the pioneering study of Oberdörster showed that C_{60} fullerenes were able to

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Product categories	2006	2008	2011
Health and fitness	120	369	738
Home and Garden	25	69	209
Automotive	10	35	126
Food and beverage	25	68	105
Cross cutting	10	12	82
Electronics and computers	30	51	59
Appliance	10	22	44
Goods for children	5	17	30

TABLE 1 The increase in "nanotech" products, according to different categories
Source: Project on Emerging Nanotechnologies, 2011

induce changes in the brain of some fishes already at very low aquatic exposure level[2]. Then, it became clear that the environmental adverse effect of NPs should not be overlooked and, therefore, NPs started to be considered as a novel class of environmental contaminants whose ecotoxicity necessarily should be evaluated.
Ecotoxicology is the science studying the contaminants effects on the biosphere constituents. Albeit relatively new, ecotoxicological research is rapidly developing due to concern induced by the industrial development. Ecotoxicology has therefore become an important part in the environmental and ecological risk assessment and in the definition of environmental policies. As a matter of facts, differently from analytical chemistry approaches, ecotoxicological tests integrate all toxic signals, thus adding toxicity-based criteria to the currently adopted policies for a more comprehensive evaluation of the environmental hazard.
The assessment of risks related to nanomaterials (NMs) represents, however, a real challenge for toxicologists. Reducing the particle size down to the nanoscale changes the physicochemical properties of the materials opening the way to novel and often exciting properties and engineering possibilities. On the other hand, it is not unreasonable to assume that these size-dependent alterations may also result in different and unpredictable interactions with biological systems as compared with their bulk counterparts. It is in fact commonly accepted that the high specific surface area, resulting in an enhanced reactivity of NMs, may also lead to an increased bioavailability and toxicity[3]. Hence, accurate NMs characterizations are essential to provide the basis for understanding the nano-induced properties and their biological effects. In this respect, three key elements of NPs toxicity screening strategies have been outlined[4]: (i) the physicochemical characterization (size, surface area, shape, solubility, aggregation) and the elucidation of biological effects involving (ii) in vitro and (iii) *in vivo* studies. These three key elements were formulated mainly from the point of view of potential effects of NPs on humans. When the whole ecosystem is concerned, the problem is clearly far more complicated. Although there is already a remarkable amount of toxicological information concerning NPs (obtained at various biological levels, from *in vitro* cell cultures to in vivo studies on rodents), ecotoxicological data on NPs are just emerging.
It is clear that the evaluation of toxicological impact of nanomaterials needs to bridge the fields of nanoscience and toxicology with a holistic approach. Collaborative effort and integrated approach, to date, have produced the most instructive studies in this new field. In the light of these considerations, in the ENEA – Portici research centre, expert researchers in ecotoxicology and in na-

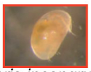
Test organism	Test endpoint	Reference
 *Lepidium sativum*	seed germination root elongation 72 h	EPA, 1996; OECD, 2003
 *Hetrocypris incongruens*	6d growth mortality	Chial & Persoone 2002
 *Folsomia candida*	reproduction rate 28 d avoidance test 100 min	ISO 11267 (1999) Aldaya *et al.* 2006
 *Vicia faba*	Micronucleus frequency 96 h	Kanaya *et al.* 1994

TABLE 2 Toxicity test battery for terrestrial environment
Source: ENEA

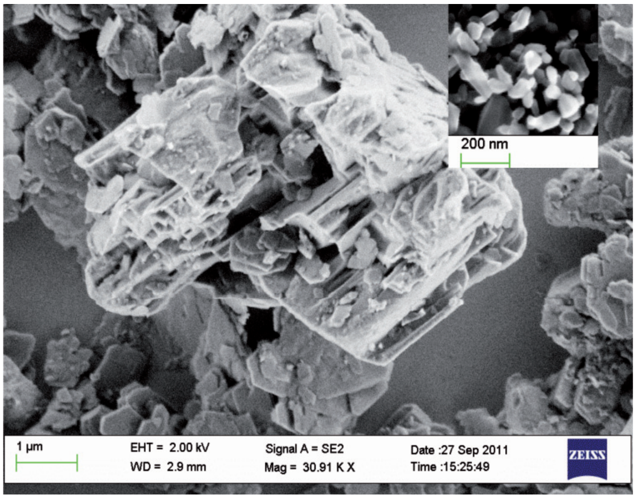


FIGURE 1 SEM image of a ZnO contaminated soil sample ($mg_{Zn\ n}$ kg_{soil}^{-1}). Inset: SEM image of pure ZnO NPs
Source: ENEA

nomaterials synthesis, processing and characterization gathered their efforts and formed an interdisciplinary/interactive group since 2007 with the ambition to contribute to the assessment of the ecotoxicological impact of nanomaterials in the environment[5-10]. The focus is on the investigation of NMs impact on aquatic (freshwater and seawater) and terrestrial ecosystems. Acute, chronic and genotoxic effects on test organisms are investigated by standard methods and newly designed methodologies while the studies regard the main trophic levels in order to get a full picture of the NMs impact. By integrating the different competences coming from the UTTP-MDB and UTTP-CHIA laboratories, the group gained a good expertise in the physicochemical characterization of NMs not only in the native state but also in the relevant environmental matrices (soil and aqueous matrices), which is of the utmost importance in this field and in the adaptation and development of toxicity assays that could properly fit with the specific issues related to the investigation of NMs.

As it is evident from Table 1, "Health & Fitness" is the most important among the categories of nanoscale products containing nanoparticles (more than 50%) with the "Personal Care" subsector covering, alone, around 20% of the nanotech goods. The materials most commonly reported in their nanophase in the products description are silver (313 products), used for its antimicrobial

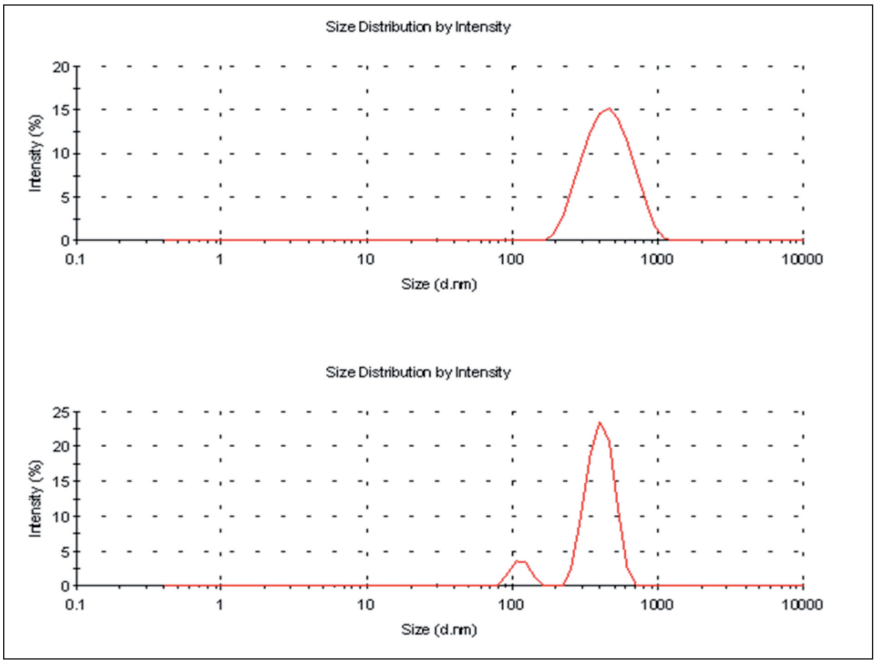


FIGURE 2 Particle size distribution in water extracts of standard OECD soil (a) and in ZnO NP contaminated OECD soil (b)
Source: ENEA

properties, Titanium dioxide (59) and zinc oxide (31), similarly largely used in the production of sunscreens and baby care products.

In spite of this large diffusion, relatively very few studies have reported on the evaluation of the environmental impact of nano-ZnO in soils and waters. We therefore focused our recent investigations on this topic. Herein we report our main findings related to the toxic effect of ZnO in its nano-form, either in soil and in water.

Soil

We investigated the toxic and genotoxic impact of ZnO nanoparticles towards several terrestrial organisms: plants (*Lepidium sativum, Vicia faba*), crustaceans (*Heterocypris incongruens*), insects (Folsomia candida)[5]. In Table 2, the toxicity test battery used for this kind of investigations is reported.

The effects of ZnO NPs were also evaluated with respect to free metal ions (as $ZnCl_2$) which are considered as the source for the toxic action of the corresponding metal oxides nanoparticles.

To ensure the nanostate in our experiment, we spiked the soil by dry mixing the nano-powder to it. This procedure preserves the NP pristine size and leaves the dispersion state of the NPs unchanged after the wetting procedure of the soil carried out for toxicity tests, as confirmed by SEM and DLS characterizations (Figures 1, 2).

In Figure 2, the DLS analysis of spiked soil aqueous extracts showed the presence of dispersed particles with an average size of 103 nm, which can be ascribed, by comparison with DLS analysis of clean OECD soil extract, to the ZnO NPs.

The most sensitive organism to ZnO NP exposure was *H. incongruens* (Figure 3). Actually, the presence of ZnO dispersed NPs in the soil caused a lethal effect (100% mortality) after 6 days of exposure. Under the same exposure conditions, $ZnCl_2$ spiked soil (that is soluble Zn) only caused moderate, acute (21%) and chronic (34%) effects. It has been already reported that the soluble zinc ions are able to exert toxic effects against the *P. subcapitata*[11] (used as feeding organisms in our experiment) and this toxic action may, in turn, affect the overall toxicity against H. incongruens resulting, in our case, in acute and chronic responses up to 21% and 34%, respectively. In order to explain the 100% mortality observed by the ZnO exposure, we are then forced to assume some other toxic mechanism involving a more specific interference with some kind of vital processes of the ostracod amplified by the nanodimension.

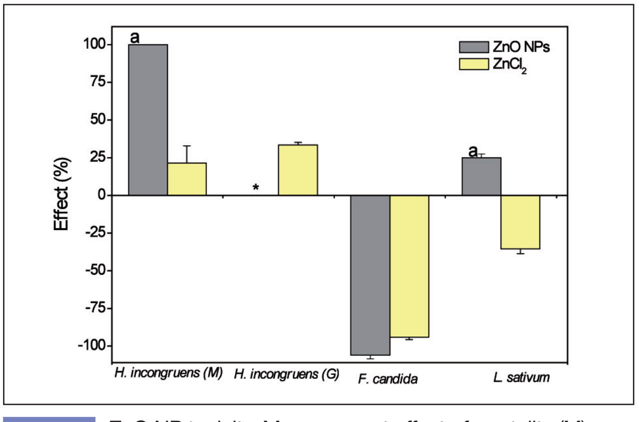


FIGURE 3 ZnO NP toxicity. Mean percent effect of mortality (M) and body growth (G) measured at Zn concentration of 230 mg kg^{-1} d.w., tested both as ZnO NPs and $ZnCl_2$. (a) indicates statistically significant difference with $p<0.05$ between the two treatments; (*) no data could be measured because of 100% mortality

Source: ENEA

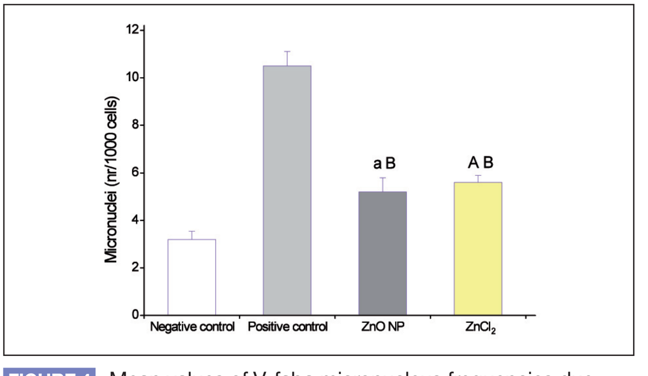


FIGURE 4 Mean values of V. faba micronucleus frequencies due to exposure to ZnO NP and $ZnCl_2$ spiked soils. OECD soil was used as negative control. Positive control was prepared by standard soil saturated with $K_2Cr_2O_7$ solution (10 mg l^{-1}) . Statistically significant differences between each treatment and the negative control have (a) $p<0.05$ and (A) $p<0.01$; as for positive control differences were statistically significant with (B) $p<0.01$

Source: ENEA

Plants were less sensitive to the exposure treatments. L. sativum seeds showed a 100% germination (no effect with respect to control) with both soil contaminants, whereas the root elongation was affected by the exposure to ZnO NPs and soluble Zn in different ways. As can be seen in Figure 3, ZnO NPs spiked soil exerted a moderate toxic effect, while $ZnCl_2$ spiked soil even produced a 35% biostimulation. This biostimulation can be most likely addressed to a hormetic effect. As a matter of fact, the OECD standard soil is devoid of Zn and, as this metal is an essential element for many plants, a certain amount of promptly available ionic Zn might result in a biostimulation of the terrestrial organism[12].

The collembolan reproduction was not affected by the presence of Zn regardless of its form. Actually, both ZnO NPs and $ZnCl_2$ spiked soils produced a clear biostimulation (106% and 94%) with respect to the control soil. Once again, this is not surprising since the observed effect could be the result of specific needs of the exposed organisms in the control soil. These results highlight that, when assessing the environmental risks of essential metals such as zinc, both deficiency and toxicity levels should be, in fact, taken into account[13,14].

V. faba micronucleus test revealed slight genotoxic effects with both spiked soils (Figure 4).

The observed effects may come from different interaction pathways of the tested materials: soluble Zn ions can easily penetrate directly into meristematic cell membranes of *V. faba* while the ZnO NPs can penetrate the cell walls through a mechanical action[15].

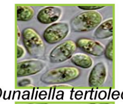
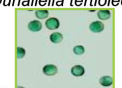
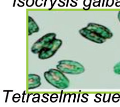
Test organism	Test endpoint	Reference
 *Dunaliella tertiolecta*	algae population growth 96 h	US EPA, 1985
 *Isocrysis galbana*		
 *Tetraselmis suecica*		
 *Artemia salina*	mortality 48h	IRSA-CNR, 1997
 *Paracentrotus lividus*	embryos development 72 h	US EPA, 1995

TABLE 3 Toxicity test battery for marine environment
Source: ENEA

Summarizing, we have reported the evidence of toxic effects of ZnO NPs towards different terrestrial organisms. Although most of the results reported in literature call for the soluble fraction of the ZnO NPs (i.e., the Zn^{2+} ion) to explain the ecotoxic actions, we showed that for some organisms ZnO NPs exert a higher toxic effect in their insoluble form compared to that of the same amount of ionic zinc.

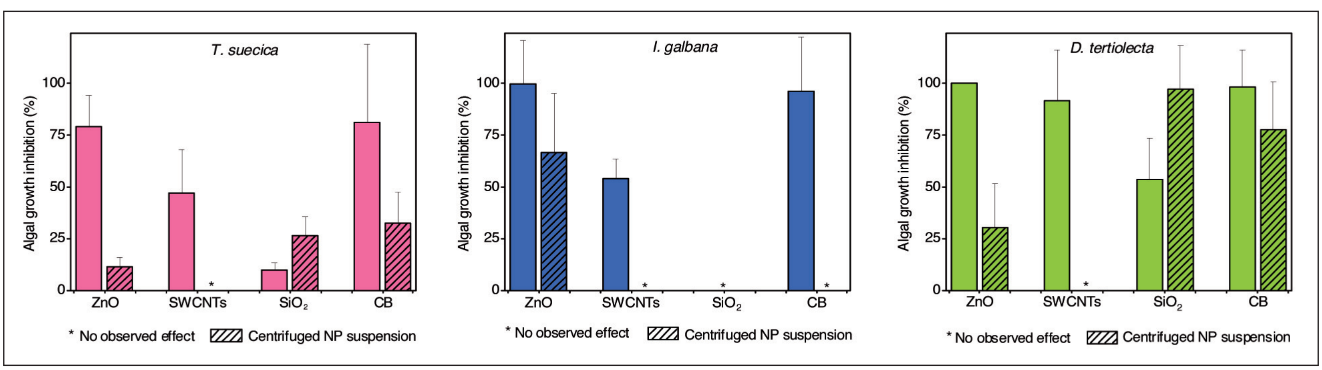


FIGURE 5 Mean percent algal growth inhibition upon 96h exposure to seawater dispersions of ZnO, SWCNTs, SiO_2 and CB nanoparticles. Error bars are also shown
Source: ENEA

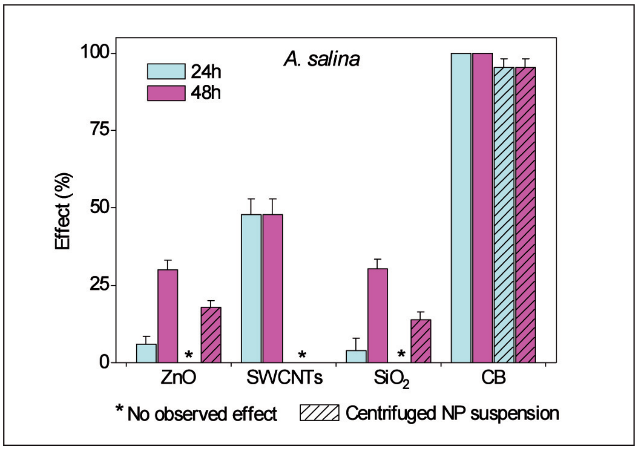


FIGURE 6 Mean percent effect on A. salina exposed to NPs samples. Error bars are also shown
Source: ENEA

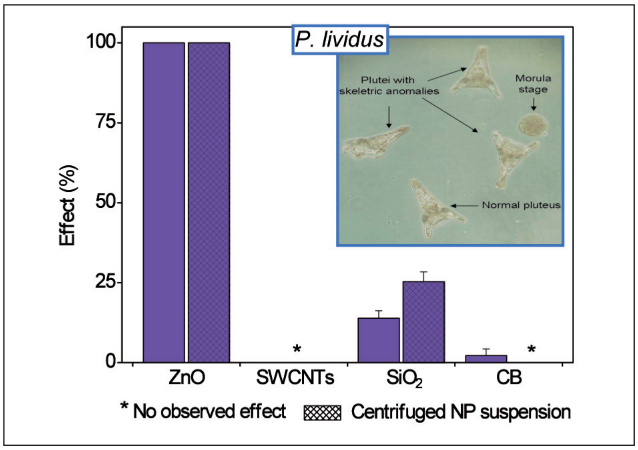


FIGURE 7 Mean percent effect on sea urchin embryos. Error bars are also shown
Source: ENEA

Thus, the NPs toxic action can be linked to a chemical effect and/ or to stress or stimuli caused by the peculiar physical characteristics of the nanostate.

Sea Water

Coastal systems are likely to be the ultimate sink for any nanomaterial, more or less deliberately released into the environment[16]. The fate and behaviour of NPs in seawater strongly depend on different physicochemical characteristics that could affect NPs aggregations. It is well-known that NPs tend to aggregate in aquatic environments to form micrometer-sized particles and it is likely that this state of dispersion may affect the influence of particle size, shape and surface properties on their ecotoxicity[17,18].
NPs and NP aggregates could represent a risk both for pelagic and, after deposition in sediment, for benthic species. Despite this concern, only a few studies regarding the ecotoxic effect of NPs upon marine organisms have been so far accomplished.
Our research work, about this peculiar environmental matrix, started with the evaluation of adverse effect of nano ZnO on marine organisms with different biological complexity (sea urchins, crustaceans and algae SW Table 3) also with respect to other different kinds of NPs such as Silicon dioxide (SiO_2), Carbon black and Carbon nanotubes (CNTs).

In Figure 5, the mean algal growth inhibition upon NP exposure is summarized.
The selected algae got comparable responses upon NP exposure, with *D. tertiolecta* being the most sensitive organism. Among the tested nanomaterials, only SiO_2 exerted a very low effect on the growth inhibition of *T. suecica* and no effect at all on *I. galbana*. The removal of large aggregates decreased the toxic effect likely because of an appreciable decrease of the concentration of suspended particles in the seawater samples. This was particularly evident in the case of the exposure of all the tested organisms to SWCNTs. In this case the toxic effects shown by SWCNTs uncentrifuged samples vanished when centrifuged seawater sample were supplied to the algae.
On the whole, carbon based nanoparticles have shown the highest effects on crustaceans (Figure 6).
In fact, exposure of *A. salina* to CB and SWCNTs suspensions caused a 50% and 95% mortality after the first 24 h, respectively. The toxic effect of CB NPs was so marked that the response was equivalent with both samples, centrifuged and uncentrifuged. Mild effects were observed, instead, for ZnO and SiO_2 NPs in the uncentrifuged suspensions after 48 h.
As shown in Figure 7, echinoids were very sensitive to ZnO NPs. The SiO_2 showed only a moderate toxic effect while SWCNTs and CB did not show any toxic effects on sea urchin embryos.

NPs effects upon marine phytoplankton is a necessary step to predict their potential impact on coastal marine food webs and on the whole ecosystems that they support. Hence, following these preliminary results we focused the investigation on the ecotoxicological behaviour of ZnO. To this aim, we recorded dose response curves for exposure to nano-ZnO and calculated for the first time its main toxicological parameters[6]. Bulk-ZnO dose response curves were also measured in order to check any specificity in the nanosized material with respect to its bulk counterpart. The findings were finally compared to the toxic effects of Zn^{2+}, to specifically investigate the metal ions contributions to ZnO toxicity. The results show that nano-ZnO is more toxic (EC50: 2.42 (0.97-5.36) mg L-1, NOEC: 0.01 mg L^{-1}) than its bulk counterpart (EC50: 4.45 (3.45-5.98) mg L^{-1}, NOEC: 1 mg L^{-1}). Cross-referencing the toxicity parameters calculated for ionic zinc (EC50: 0.65 (0.36-0.70) mg L^{-1}, NOEC: 0.01 mg L^{-1}) and the dissolution properties of the ZnO, it can be noticed that the toxicity of nano-ZnO cannot be ascribed to zinc ions exclusively. At the same time, growth rates of *D. tertiolecta* were not significantly affected by nano-ZnO exposure.
It is clearly evident thereby that a different toxicological pathway, relying on a nano-size effect, has to be assumed also in this case to understand the considerable differences between nano and bulk ZnO behaviours.

Conclusions

In conclusion, our findings suggest that the size of ZnO dispersed particles determines their bioavailability and overall toxicity. The true operating mechanism is however still undisclosed. Therefore, due to the wide diffusion of nano-ZnO in many commercial products, our results suggest that nanomaterials may actually have an adverse impact on the environment and thus great care should be adopted when dealing with nanomaterials in their whole life cycle.

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