

Investigation of ZnO nanoparticles' ecotoxicological effects towards different soil organisms

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Received: 21 June 2010 / Accepted: 18 November 2010 / Published online: 30 November 2010
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Abstract

Introduction Nanomaterials have widespread applications in several industrial sectors. ZnO nanoparticles (NPs) are among the most commonly used metal oxide NPs in personal care products, coating and paints. However, their potential toxicological impact on the environment is largely unexplored.

Materials and methods The aim of this work was to evaluate whether ZnO nanoparticles exert toxic and genotoxic effects upon terrestrial organisms: plants (*Lepidium sativum*, *Vicia faba*), crustaceans (*Heterocypris incongruens*), insects (*Folsomia candida*). To achieve this purpose, organisms pertaining to different trophic levels of the soil ecosystem have been exposed to ZnO NPs. In parallel, the selected soil organisms have been exposed to the same amount of Zn in its ionic form (Zn^{2+}) and the effects have been compared.

Results The most conspicuous effect, among the test battery organisms, was obtained with the ostracod *H. incongruens*, which was observed to be the most sensitive organism to ZnO NPs. The root elongation of *L. sativum* was also mainly affected by exposure to ZnO NPs with respect to $ZnCl_2$, while collembolan reproduction test produced similar results for both Zn compounds. Slight genotoxic effects with *V. faba* micronucleus test were observed with both soils.

Conclusion Nanostructured ZnO seems to exert a higher toxic effect in insoluble form towards different terrestrial organisms with respect to similar amounts of zinc in ionic form.

Keywords ZnO nanoparticles · Soil · Toxicity test · Genotoxicity

1 Introduction

A nanoparticle may be defined as any solid state material that has at least one dimension below 100 nm and whose properties are different from its bulk counterpart (Auffan et al. 2009). Due to their novel and remarkable properties, nanomaterials are finding widespread applications in several industrial sectors. This poses an urgent need for the assessment of their potential toxicological impact on human health and environment. Indeed, several papers have reported on its toxicology in relation to human health (Auffan et al. 2009). In contrast, the effect of nanoparticles (NPs) released in the environment is still largely unexplored although some reviews have been already published (Baun et al. 2008; Kahru et al. 2008; Ruffini Castiglione and Cremonini 2009). Two different mechanisms compete to control the NPs toxic action (Brunner et al. 2006): (1) a chemical effect based on the chemical composition, e.g., release of (toxic) ions; and (2) stress or stimuli caused by the surface, size and/or shape of the nanoparticle itself. These stimuli can be either due to a mechanical hindrance to biological functions or to a different interaction of the chemical compound in the nanostructured form with the biological environment. In fact, the peculiar properties of the nanostate, with special attention to the atypical surface structure and reactivity, may modify the biophysico-

Responsible editor: Vera Slaveykova

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chemical interactions of these materials with biological environment, enhancing processes such as dissolution, redox reaction, generation of reactive oxygen species, affecting the NPs reactivity towards the environment (Nel et al. 2009; Carlson et al. 2008; Xia et al. 2006). Thus, when investigating the toxicity of a compound in relation to its nanometric nature, it is important to verify which is the actual size of the particles dispersed in the testing media.

In this frame, ZnO are among the most widely used NPs since they have applications in a large variety of sectors ranging from personal care products to coatings and catalysts in environmental remediation (Choopun et al. 2009; Kamat and Meisel 2003; Wang 2004).

The toxicity of ZnO nanoparticles in freshwater has been thoroughly investigated and the EC_{50} and LC_{50} levels have been registered for several aquatic organisms: for *Vibrio fischeri* EC_{50} is 1.9 mg l^{-1} ; for *Daphnia magna*, LC_{50} is 3.2 mg l^{-1} ; for *Tamnocephalus platyurus*, LC_{50} is 0.18 mg l^{-1} (Heinlaan et al. 2008); and for the freshwater alga *Pseudokirchneriella subcapitata*, the estimated 72-h EC_{50} value is as low as 0.07 mg l^{-1} (Franklin et al. 2007). ZnO nanoparticles exposition has been found to induce effects on humans as well. For instance, they can cause gastroduodenal corrosive injury (Liu et al. 2006) and pulmonary toxicity (Moos et al. 2010). In most cases, soluble zinc ions (Zn^{2+}) from ZnO seem to be the main source for the (eco)toxicity (Aruoja et al. 2009; Heinlaan et al. 2008). For *T. platyurus*, the toxicity is attributed to the soluble, dissociated Zn ions, and the LC_{50} and NOEC values for *D. magna* are up to threefold lower for ZnO NPs than for bulk ZnO, suggesting a clear toxic effect of the nano ZnO due to the peculiarity of the nanometric size.

In comparison to freshwater, much less data have been reported on the NPs toxicity effects in soils although the soil solid phase is the primary, large sink for any human waste.

As far as elemental zinc is concerned, it is well known that it is one of the most important soil contaminant and furthermore a number of key processes are likely to affect its fate and availability in this environment. Nevertheless scarce data have been published on the effects produced by zinc based nanomaterials in soil. To date, phytotoxic effects of nanomaterials have been mainly investigated (Lin and Xing 2007). Goodman et al. (2004) have reported a clear reduction in seed germination of corn and in root growth of corn, cucumber, lettuce, radish, mustard rape, and ryegrass upon exposure to functionalized gold NPs.

In this study, we investigated the ZnO nanopowder toxic effect on different terrestrial organisms. Since most of the cited literature agrees on the involvement of the ion Zn^{2+} in the toxic action of zinc based nanoparticles, in this work the impact of ZnO NPs on terrestrial organisms has been investigated and compared to that of the same amount of ionic zinc.

As no single test or species of living organism shows uniform sensitivity to all chemical compounds, a biotest battery with different sensitivity profiles is often recommended and used to perform the ecotoxicological assessment. In addition, due to the complexity of the ecosystems, the toxicological hazard assessment is more informative/predictive if the battery involves organisms of different trophic levels (Blaise 1998). According to these general criteria, in this study a battery of toxicity contact tests with different organisms: plants (*Lepidium sativum*), crustaceans (*Heterocypris incongruens*), insects (*Folsomia candida*), together with a genotoxicity test with plant seeds (*Vicia faba*), were performed.

2 Materials and methods

$ZnCl_2$ with purity >98% was purchased from Sigma-Aldrich (CAS Number: 20,808-6). ZnO nanopowder was purchased from Sigma-Aldrich (CAS Number: 1314-13-2), with a nominal primary particle size of less than 100 nm (i.e., $r_p \leq 50 \text{ nm}$).

Pure water (conductivity: $0.056 \mu\text{S cm}^{-1}$; TOC <5 ppb) was prepared with Milli-Q Gradient A10 system, Millipore. OECD (Organisation for Economic Co-operation and Development) standard soil was purchased from ECOTOX LDS srl (Milan, Italy).

A $ZnCl_2$ stock solution was prepared at a concentration of 10 g l^{-1} in pure water.

ZnO nanopowder (10 mg) was finely dispersed in 35 g of OECD dry standard soil. The 10 g soil aliquots for toxicity assays were sampled from the whole solid mixture through a Fritsch rotary sample divider Laborette 27.

The specific surface (SS) of both the soil samples and ZnO NPs was analyzed using the Brunauer, Emmett and Teller (BET) method on a Quantachrome, Autosorb-I instrument, recording N_2 adsorption/desorption isotherms at 77 K (Brunauer et al. 1938).

Aqueous extracts of soil samples were obtained by mixing 2.5 g of soil with 15 ml of pure water and vigorously stirring the mixtures for 30 min.

Measurements of particles size were performed with a Zetasizer Nano system (Malvern Instruments, U.K.). Unfiltered aqueous extracts were placed in clean disposable cuvettes and measured, at least in triplicate, at 25°C. At first, the presence of very large sedimenting particles negatively affected the measurements. After particles settling, instrument's internal quality criteria were passed and data were recorded. All tests were conducted at least in triplicate.

The nanoparticles dissolution was assessed by measuring and comparing the Zn content in unfiltered aqueous extracts and in samples filtered through an alumina Whatman

Anotop filter 0.02 μm . Samples were diluted tenfold with pure water prior to the inductively coupled plasma-mass spectrometry (ICP-MS) analysis. Measurements were performed on a spectrometer ICP-MS Elan 6000 (Perkin-Elmer) equipped with a cross-flow nebulizer.

SEM analysis was performed, on a LEO 1530, over soils samples with two different concentration of zinc oxide, at 0.03% and 10%. Small soil aliquots were wetted with few millilitres of pure water and the obtained suspensions casted over a clean silica wafer and finally dried onto a hot plate.

2.1 Experimental design

To compare the toxicity due to the different chemical and structural forms of zinc, a battery of toxicity tests was performed on artificial standard soil (OECD 2000) samples spiked with ZnO NPs and ZnCl_2 , both containing the same Zn amount. A screening dose of Zn (i.e., 230 $\text{mg kg}_{\text{soil}}^{-1}$) was used for spiking the dry soil. This concentration was chosen to satisfy two requirements: (1) for NPs, the Zn concentration is well above the solubility of the oxide, so that ZnO can be considered as only negligibly dissociated into ions (Zn^{2+} and/or $\text{Zn}(\text{OH})^+$) even in wetted soil matrices; (2) for ZnCl_2 testing, the metal ion concentration is capable of producing clear, marked effects on the selected organisms for toxicity tests (Aramba et al. 1995; Lock and Janssen 2003).

According to the OECD guidelines, standard artificial soil, composed by sphagnum peat (10%), kaolin clay (20%) and quartz sand (70%), was used both as control soil and as matrix for Zn contamination. For spiking of the soil with ZnO and ZnCl_2 , two different routes were followed: zinc oxide nanopowder was finely dispersed in the dry solid matrix, while the zinc chloride was administered as aqueous solution to the dry soil. The use of different administration routes was aimed to supply the contaminants in the specific structural/chemical form to be tested (i.e., nanopowder in its primary particle size and zinc chloride in its ionic, dissociated form).

Ten milligrams of zinc oxide nanopowder was directly added and thoroughly mixed with 35 g dry weight (d.w.) of the standard soil to obtain a concentration of 286 mg kg^{-1} d.w. (corresponding to 230 mg kg^{-1} d.w. of Zn). In order to obtain representative samples of this contaminated soil to be employed in toxicity assays, soil aliquots (10 g) were collected by sampling them from a stream of the solid mixture, taking further care of sampling the entire cross section of the stream for many times. Different aliquots of pure water were then added to the spiked soil in agreement with the different assays to be performed. The same nominal zinc concentration was reached by adding a ZnCl_2 aqueous solution to the soil. The spiked soil obtained was

then wetted, for the test, taking into account the water added with the ZnCl_2 solution.

2.2 Characterization of ZnO spiked soil

If an ensemble of NPs is dispersed in an external matrix, under the assumption that no interaction occurs between the NPs and the matrix itself, the dispersion specific surface (SSd) can be considered as the weighted mean of the external matrix and the NPs specific surfaces, SSem and SSnp, respectively:

$$\text{SSd} = (1 - \chi)\text{SSem} + \chi\text{SSnp} \quad (1)$$

where χ is the ratio of the NP weight over the dispersion weight.

Rearranging Eq. 1, it turns out that:

$$\text{SSd} = (\text{SSnp} - \text{SSem})\chi + \text{SSem} \quad (2)$$

Therefore, if SSd is measured vs. χ and the relation is found to be linear, then the NPs ensemble and the external matrix can be considered non-interacting and, once SSnp is evaluated from the best fit, the average NP radius in the dispersion, r_p can be calculated by means of Eq. 3 (Roelofs and Vogelsberger 2004).

$$\text{SSnp} = N*a_0 = 3/r_p*\rho \quad (3)$$

where $a_0 = 4*\pi*r_p^2$ is the surface of a single NP (assumed spherical for simplicity), r_p is the NP radius and N is the total number of NPs per unit of mass.

In Table 1, the dispersion specific surfaces are reported vs. χ in our case, in which the external matrix is the standard soil and the NPs ensemble consists of ZnO nanoparticles.

In our experimental conditions, the relative error on SSd is less than $\pm 0.2\%$. It is easily found that data follow very accurately a linear fit and, from the slope and the intercept, an NP specific surface (SSnp) of 9.89 m^2/g is extracted. Substituting this value into Eq. 3, we find: $r_p = 53$ nm, which is in good agreement with the actual radius of ZnO NPs used in our experiments. We can therefore conclude that ZnO NPs do not form aggregates and that the ensemble chemical and physical properties are the result of the

Table 1 Weight ratio of NPs in soil dispersions χ and the specific surface area of soil samples measured via BET method

χ (%)	SSd (m^2/g)
0	0.78
0.1	1.19
0.2	2.62
0.2	2.49 ^a
1	9.9

^a Wetted sample

superimposition of the chemical and physical properties of each of the ZnO NP comprising the ensemble itself.

In addition, soil samples, both contaminated and not, were extracted with pure water in order to investigate the ZnO solubility under the experimental conditions and to characterize the size distribution of suspended matter in the aqueous extracts. The tests were performed over 2.5 g aliquots of OECD standard soil and contaminated soil suspended in 15 ml of pure water. The extracts were rested to settle and the supernatant was collected for particle sizing via dynamic light scattering (DLS) and Zn analysis via ICP-MS. Aqueous extracts of soil samples have pH 6.2–6.5 values.

2.3 Toxicity tests

2.3.1 Plants

Seed germination and root elongation test According to EPA (1996) and OECD (2003) guidelines, ten *L. sativum* seeds were exposed to 10 g of contaminated soil and control soil (OECD) and wetted with 5 ml of deionized water. After incubation in darkness ($25\pm 2^\circ\text{C}$, 72 h), the germinated seeds were counted and the root lengths measured. The results were expressed as percent effect with respect to the control (percent effect).

***V. faba* micronucleus test** Five *V. faba* seeds were exposed to 20 g of contaminated soil and control soil, both saturated with 6 ml deionized water. OECD soil was used as negative control while positive control was prepared by saturating OECD soil with 6 ml of a $10\text{ mg l}^{-1}\text{ K}_2\text{Cr}_2\text{O}_7$ solution. After incubation in darkness ($20\pm 1^\circ\text{C}$, 96 h), according to Kanaya et al. (1994) the primary roots were cut, fixed and then stained by the Feulgen technique. Squash preparations were produced in 45% acetic acid and the slides made permanent in Histovitrex (Carlo Erba, Milan, Italy). Two hundred cells per tip were scored in order to evaluate micronucleus frequency.

2.3.2 Crustaceans

***H. incongruens* growth and mortality test** Following the Chial and Persoone (2002a, b), ten freshly hatched ostracods were exposed to 1 g of contaminated soil and control soil (OECD) previously added with 4 ml of standard solution (EPA Standard Freshwater). One millilitre of algal suspension (*P. subcapitata* 10^7 cells ml^{-1}) was added to each well as food supply. After incubation in darkness ($25\pm 2^\circ\text{C}$, 6 days), mortality rates were recorded and the lengths of organisms that survived were measured. The results are expressed as mortality and growth percentage with respect to the control.

2.3.3 Insects

***F. candida* reproduction test** Following ISO 11267 (1999), ten juvenile collembolans (*F. candida*) aged 10–12 days were exposed to 30 g of control and spiked soils saturated with 9 ml of deionized water and incubated under controlled conditions (light/dark cycle 16/8 h; $20\pm 2^\circ\text{C}$). After 28 days of exposure, during which insects were fed weekly with 2 mg of dry yeast, test chambers were floated to allow survived adult and neonate count and to evaluate mortality and reproduction endpoints.

***F. candida* avoidance test** Following Aldaya et al. (2006), 7.5 g of standard soil as control and 7.5 g of contaminated soil, both saturated with 2.25 ml of deionized water, were placed in sterile polystyrene Petri dishes (55 mm diameter, 10 mm height), leaving a 2-mm space line between the soils. At the centre of the line an individual of *F. candida* was deposited and its position was recorded every 20 min up to 100 min. The test was performed over five replicates. During the observation period, the Petri dishes were kept under fluorescent light at $20\pm 2^\circ\text{C}$. A blank experiment using control soil at both sides of the Petri dishes was performed to check for any light gradient effect. The five counts over the 100-min observation period for each Petri dish was used to calculate the average position of a single individual.

2.4 Statistic analysis

All toxicity test results are expressed as mean \pm standard error (SE). The toxicity data obtained for both ZnO NP and ZnCl_2 treatments were analyzed with Student's *t*-test using the software SigmaStat v. 3.5. Levels of $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively.

3 Results and discussion

The BET characterization of the spiked soil samples indicate that the ZnO NPs dispersed in the soil are still in their pristine state and no aggregation has occurred. Data in Table 1 show that the addition of ZnO NPs to standard soil gives rise to a linear increase in the specific surface area with the NPs weight fraction for the contaminated soil, while the calculated BET radius of the NP itself remains unchanged. The adopted spiking procedure seems therefore to preserve the NP size. The dispersion state of the NPs remains unchanged after the wetting procedure of the soil itself, carried out in the toxicity tests, as could be observed by measuring the SSd in the wetted soil. SEM analysis of

the contaminated soil sample (i.e., $230 \text{ mg}_{\text{Zn}} \text{ kg}_{\text{soil}}^{-1}$, corresponding to 0.03 wt.%) is not very informative. In fact, due to the very low concentration of nanoparticles, even after a very careful examination of this soil sample, it was not possible to identify any particle, agglomerate or mass portion that could be ascribed to the ZnO (Fig. 1). It is interesting to note that a bimodal distribution of particles is observed only in the aqueous extract of a 10 wt.% of ZnO contaminated soil, for which the DLS analysis show well resolved peaks at 470 and 103 nm. Other test soils (i.e., 0 and 0.03 wt.%) have shown monomodal distributions. Therefore, the peak at 103 nm can be ascribed to the ZnO NPs, whereas the other peak is assigned, by comparison, to the soil component.

Among the test battery organisms, the most striking effect of ZnO NP spiked soil was obtained with the ostracod *H. incongruens*, which was observed to be the most sensitive organism to ZnO NPs (Fig. 2). *H. incongruens* is an ostracod recently introduced as a test organism for soil toxicity assessment (Chial and Persoone 2002a, b) which allows handlers to evaluate both acute and chronic endpoints. ZnO NPs spiked soil exerted a lethal effect (100% mortality) after 6 days of exposure. On the contrary, ZnCl₂ spiked soil (soluble Zn) caused only moderate, acute (21%) and chronic (34%) effects. The noticeable difference between the toxic action exerted by ZnO NPs and ionic zinc against these organisms could be ascribed to an interference with some vital processes of the oxide conveyed by the nanodimension. According to Franklin et al. (2007), a similar, indirect effect through food depletion, i.e., algae *P. subcapitata*, due to soluble zinc, may be taken into account for both the experiments. This study shows that the 72-h IC₅₀ values against *P. subcapitata* for ZnO bulk, ZnO nano and ZnCl₂ range from 60 to 69 $\mu\text{g l}^{-1}$, and that the toxic action can be attributable to the solely dissolved zinc.

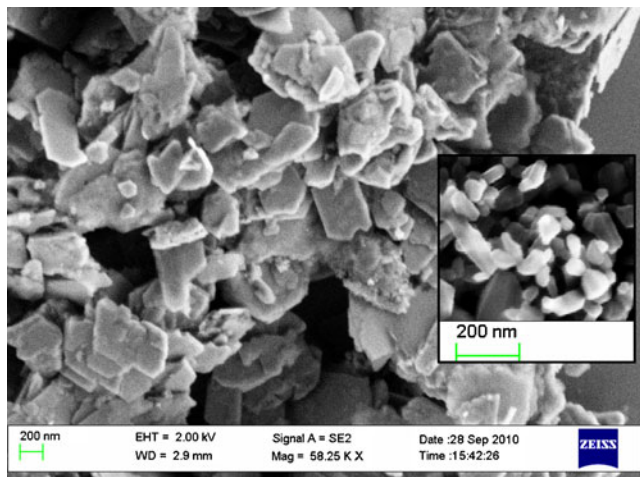


Fig. 1 SEM image of a ZnO contaminated soil sample ($230 \text{ mg}_{\text{Zn}} \text{ kg}_{\text{soil}}^{-1}$). Inset: SEM image of pure ZnO NPs

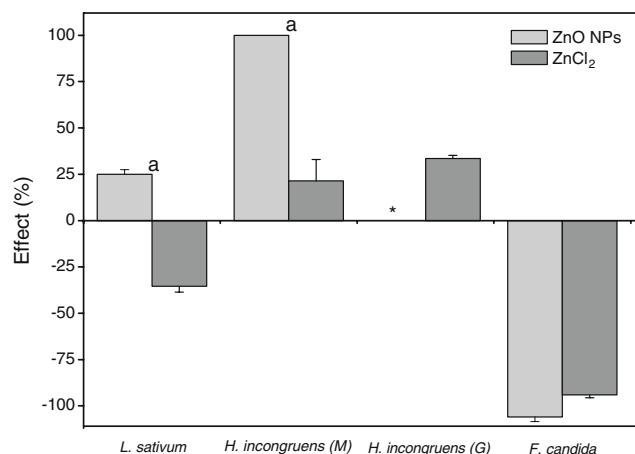


Fig. 2 ZnO NP toxicity. Mean percent effect of mortality (M) and body growth (G) measured at Zn concentration of 230 mg g^{-1} d.w., tested both as ZnO NPs and ZnCl₂. (a) Statistically significant difference with $p < 0.05$ between the two treatments; (*) no data could be measured because of 100% mortality

In our experiment, a much higher concentration of ionic zinc (as ZnCl₂) was administered to the soil (at 57.5 mg l^{-1}), and hence, to the feeding organism *P. subcapitata*. Analogously, the same toxic action on the green alga has to be considered in the evaluation of the ZnO NPs effects. In fact, even if the ICP-MS analysis have shown that the concentration of zinc in the aqueous extracts corresponds to less than two order of magnitude (i.e., $1.6 \text{ mg kg}_{\text{soil}}^{-1}$) with respect to the ionic zinc concentration derived by ZnCl₂ ($230 \text{ mg kg}_{\text{soil}}^{-1}$), still the total amount of zinc in the water phase ($290 \mu\text{g l}^{-1}$) exceeds the toxicity value for the total Zn observed by Franklin. Following the filtration of the raw extract with a 0.02- μm filter, thus separating the ZnO NPs from the aqueous solution, the zinc concentration is only slightly decreased to $104 \mu\text{g l}^{-1}$ that is, still higher than the IC₅₀ value already reported. Therefore, it is clear that in our experiment, the soluble zinc ions do exert toxic effects against the feeding organisms, *P. subcapitata*, and that this toxic action may, in turn, affect the overall toxicity against *H. incongruens* resulting in acute and chronic responses up to 21% and 34%, respectively. However, in order to explain the 100% of mortality observed we should hypothesize some other toxic mechanism. It has been reported, indeed, that NPs may adsorb on phytoplankton (Rhee and Thomson 1992) and onto the algal cell surface (Navarro et al. 2008) or even that adhesion of NPs aggregates to the exoskeleton of crustaceans (Baun et al. 2008) may cause physical effects and/or loss of mobility. This study is, to the best of our knowledge, the first to report the effect of NPs upon ostracods.

L. sativum seeds showed a 100% germination (no effect with respect to control) with both the soil contaminants. A clear difference between ZnO NPs and soluble Zn can be observed instead for root elongation; in particular, ZnO NPs

spiked soil exerted a moderate toxic effect, while ZnCl₂ spiked soil produced a 35% biostimulation (Fig. 2). This biostimulation can be attributed to an hormetic effect. In 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 have a biostimulation effect (Paschke et al. 2006). The ZnO nanoparticles in the contaminated soil exerted a mild toxic effect on the root elongation. This result is in agreement with the significant inhibition effect of ZnO NPs, mainly on root growth, of six plant species observed by Lin and Xing (2007), who suggested a toxic action mainly due to a purely mechanical effect based on the production of ‘holes’ into the cell walls. This hypothesis is further supported by considering that, in our case, the soil contaminant is mainly constituted by nanoparticles of ZnO in their pristine size and that its soluble fraction cannot account for the experimental observation. As a consequence, the toxic action observed can be related to the surface properties of the NPs, whereas a chemical toxic action induced by dissolution processes can be ruled out.

Zinc seemed to exhibit no adverse effect upon collembolan reproduction at the tested concentration (230 mg kg⁻¹) regardless of the form. In contrast, both ZnO NPs and ZnCl₂ spiked soils produce a clear biostimulation (106% and 94%) with respect to the control (OECD soil). This should not surprise. Zinc is an essential element, and the observed effect could be the result of exposed organisms needs, in the control soil. When assessing the environmental risks of essential metals such as zinc, both deficiency and toxicity levels should be in fact, considered (Calabrese and Baldwin 2003; Lock et al. 2001). Similar effects have also been shown to occur in the springtails *Protaphorura armata* and *Orchesella cincta* during feeding experiments (Posthuma and van Straalen 1993; van Straalen et al. 1989). It is interesting to note that a chronic zinc toxicity for *F. candida* (EC₅₀) in standard artificial soil has been found at concentration much higher than that used in our investigation (from 487 mg kg⁻¹ d.w. [Smit and Van Gestel 1998] to 900 mg kg⁻¹ d.w. [Sandifer and Hopkin 1997]). However, in short time tests with collembolans (avoidance test), ZnO NP spiked soil produced only a 16% avoidance during the 100-min observation time with respect to a 76% avoidance for ZnCl₂ probably due to oral uptake.

Slight genotoxic effects with *V. faba* micronucleus test (Fig. 3) were appraised with both spiked soils. The micronucleus frequencies observed for ZnO NPs and ZnCl₂ contaminated soils are quite similar (5.2 and 5.6 micronuclei 1000 cell⁻¹, respectively).

The *V. faba* micronucleus test has proved to be a very sensitive and useful method: micronuclei are, in fact, the result of chromosome breaks (or mitotic anomalies) that require a passage through mitosis to be recognizable.

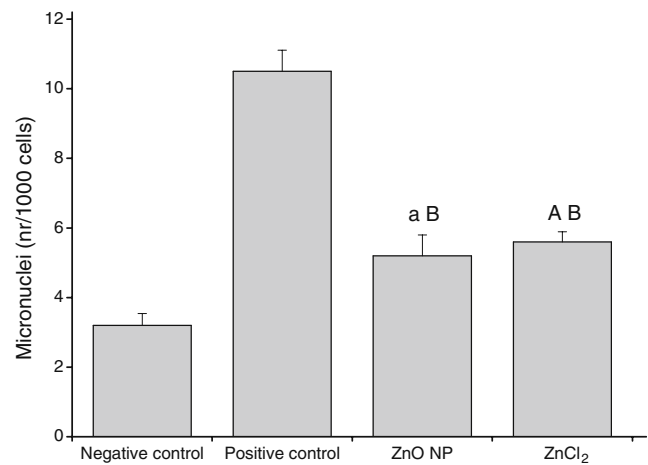


Fig. 3 Mean values of *V. faba* micronucleus frequencies due to ZnO NPs and ZnCl₂ spiked soils. OECD standard soil was used as negative control while the same standard soil saturated with K₂Cr₂O₇ solution (10 mg Γ⁻¹) was used as positive control. Differences between each treatment and the negative control were statistically significant with (a) $p < 0.05$ and (A) $p < 0.01$; for positive control they were statistically significant with (B) $p < 0.01$

Although the molecular mechanism of DNA breakage is not yet clearly understood, different toxic mechanisms have been proposed for Zn²⁺ and for ZnO NPs. Zn ions could interfere with DNA repair process similarly to what has been suggested to happen in mammals (Scicchitano and Pegg 1987; Yang et al. 1996), while it has been supposed that ZnO NPs can damage DNA by inducing lipid peroxidation and oxidative stress (Xia et al. 2006).

In this study, micronucleus tests performed with meristematic root tip cells of *V. faba* suggested the same, mild, genotoxic effect with both spiked soils. It could be presumed that the observed effects result from different interaction pathways of the tested materials: soluble Zn ions can easily penetrate directly into meristematic cell membranes while the ZnO NPs can penetrate the cell walls through a mechanical action (Lin and Xing 2007). Also, in this last case, the contribution of dissociated ionic Zn is so low that it can be neglected.

4 Conclusion

In this work we have reported the evidence of toxic effects of ZnO NPs towards different terrestrial organisms. Unlike most of the results reported in literature, which address to soluble fraction of the ZnO NPs (i.e., the Zn²⁺ ion) the (eco)toxic actions, here we show that, for some organisms, ZnO NPs exert a higher toxic effect in its insoluble form compared to that of the same amount of ionic zinc. Thus, the NPs toxic action can be linked to a chemical effect and/or stress or stimuli caused by the peculiar physical characteristics of the nanostate.

A physical interaction of ZnO nanoparticles on plant root elongation has been, in fact, observed. A high toxic action against ostracods, the most sensitive organisms, is observed and is ascribed to the ZnO nanoparticles. Besides, the genotoxic effect with *V. faba* can be linked to the nanoparticulate form, even if it is comparable to that exerted by soluble Zn. As far as the different tests utilized did not produce an univocal response, a nanoparticle-dependent toxicity has been indeed observed for the investigated NPs.

Due to the complexity of the soil ecosystem and of the considered test organisms, these findings constitute a preliminary insight into the comprehension of the biophysico-chemical interactions of nanomaterials with the biological environment. Then, for a proper evaluation of ecotoxicological risk of nanoparticles in terrestrial environment, it will be necessary to increase the battery of toxicity test utilizing more soil dwelling organisms and evaluating more different endpoints.

Acknowledgements The authors are grateful to A. Salluzzo for ICP-MS measurements and to A. De Girolamo Del Mauro and V. La Ferrara for their support in SEM analysis.

References

- Aldaya MM, Lors C, Salmon S, Ponge JF (2006) Avoidance bio-assays may help to test the ecological significance of soil pollution. *Environ Pollut* 140:173–180
- Aramba M, Bjeli S, Subakov G (1995) Acute toxicity of heavy-metals (copper, lead, zinc), phenol and sodium on *Allium cepa*, *Lepidium sativum* and *Daphnia magna*—comparative investigations and the practical applications. *Water Res* 29:497–503
- Aruoja V, Dubourguier HC, Kasemets K, Kahru A (2009) Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*. *Sci Total Environ* 407:1461–1468
- Auffan M, Rose J, Bottero JY, Lowry GV, Jolivet JP, Wiesner M (2009) Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. *Nat Nanotechnol* 4:634–641
- Baun A, Hartmann NB, Grieger K, Kusk KO (2008) Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotoxicology* 17:387–395
- Blaise C (1998) Microbiotesting: an expanding field in aquatic toxicology. *Ecotoxicol Environ Saf* 40:115–119
- Brunauer S, Emmett PH, Teller E (1938) Adsorption of gases in multimolecular layers. *J Am Chem Soc* 60:309–319
- Brunner TJ, Wick P, Manser P, Spohn P, Grass RN, Limbach LK, Bruinink A, Stark WJ (2006) In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica, and the effect of particle solubility. *Environ Sci Technol* 40:4374–4381
- Calabrese EJ, Baldwin LA (2003) Peptides and hormesis. *Crit Rev Toxicol* 33:215–304
- Carlson C, Hussain SM, Schrand AM, Braydich-Stolle LK, Hess KL, Jones RL, Schlager JJ (2008) Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. *J Phys Chem B* 112:13608–13619
- Chial B, Persoone G (2002a) Cyst-based Toxicity Tests XIII—Development of a short chronic sediment toxicity test with the ostracod crustacean *Heterocypris incongruens*: methodology and precision. *Environ Toxicol* 17:528–532
- Chial B, Persoone G (2002b) Cyst-Based Toxicity Tests XII—Development of a short chronic sediment toxicity test with the ostracod crustacean *Heterocypris incongruens*: selection of test parameters. *Environ Toxicol* 17:520–527
- Choopun S, Tubtimtae A, Santhavesuk T, Nilphai S, Wongrat E, Hongsith N (2009) Zinc oxide nanostructures for applications as ethanol sensors and dye sensitized solar cells. *Appl Surf Sci* 256:998–1002
- EPA, United States Environmental Protection Agency 712-C-96-154 (1996) Ecological effects test guidelines. OPPTS 850.4200. Seed Germination/Root Elongation Toxicity Test
- Franklin NM, Rogers NJ, Apte SC, Batley GE, Gadd GE, Casey PS (2007) Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl₂ to a fresh water microalga; *Pseudokirchneriella subcapitata*: the importance of particle solubility. *Environ Sci Technol* 41:8484–8490
- Goodman CM, McCusker CD, Yilmaz T, Rotello VM (2004) Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. *Bioconjug Chem* 15:897–900
- Heinlaan M, Ivask A, Blinova I, Dubourguier HC, Kahru A (2008) Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere* 71:1308–1316
- ISO 11267 (1999) Soil quality-inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants
- Kahru A, Dubourguier HC, Blinova I, Ivask A, Kasemets K (2008) Biotests and biosensor for ecotoxicology of metal oxide nanoparticles: a minireview. *Sensors* 8:5153–5170
- Kamat PV, Meisel D (2003) Nanoscience opportunities in environmental remediation. *CR Chim* 6:999–1007
- Kanaya N, Gill BS, Grover IS, Murin A, Osiecka R, Sandhu SS, Andersson HC (1994) *Vicia faba* chromosomal aberration assay. *Mutat Res Fund Mol* 310:231–247
- Lin DH, Xing BS (2007) Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth. *Environ Pollut* 150:243–250
- Liu CH, Lee CT, Tsai FC, Hsu SJ, Yang PM (2006) Gastrointestinal corrosive injury after oral zinc oxide. *Ann Emerg Med* 47:296
- Lock K, Janssen C (2003) Comparative toxicity of a zinc salt, zinc powder and zinc oxide to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*. *Chemosphere* 53:851–856
- Lock K, Desender K, Janssen CR (2001) Effects of metal contamination on the activity and diversity of carabid beetles in an ancient Pb–Zn mining area at Plombières (Belgium). *Entomol Exp Appl* 99:355–360
- Moos PJ, Chung K, Woessner D, Honegger M, Shane Cutler N, Veranth JM (2010) ZnO particulate matter requires cell contact for toxicity in human colon cancer cells. *Chem Res Toxicol* 23:733–739
- Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao A, Quigg A, Santschi PH, Sigg L (2008) Environmental behaviour and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology* 17:372–386
- Nel AE, Mäder L, Velegol D, Xia T, Hoek E, Somasundaran P, Klaessig F, Castranova V, Thompson M (2009) Understanding the biophysicochemical interactions at the nano–bio interface. *Nat Mater* 8:543–557
- OECD (2000) Guideline for testing chemical, Earthworm Reproduction Test (*Eisenia foetida/andrei*)
- OECD (2003) Guideline for the testing of chemicals, proposal for updating guideline 208, Terrestrial Plant Test, Seedling Emergence and Seedling Growth Test
- Paschke MW, Perry LG, Redente EF (2006) Zinc toxicity thresholds for reclamation for species. *Water Air Soil Pollut* 170:317–330

- Posthuma L, van Straalen NM (1993) Heavy-metal adaptation in terrestrial invertebrates: a review of occurrence, genetics, physiology and ecological consequences. *Comp Biochem Physiol* 106:11–38
- Rhee GY, Thomson PA (1992) Sorption of hydrophobic organic contaminants and trace metals on phytoplankton and implications for toxicity assessment. *J Aquat Ecosyst Health* 1:175–191
- Roelofs F, Vogelsberger W (2004) Dissolution kinetics of synthetic amorphous silica in biological-like media and its theoretical description. *J Phys Chem B* 108:11308–11316
- Ruffini Castiglione M, Cremonini R (2009) Nanoparticles and higher plants. *Caryologia* 62:161–165
- Sandifer RD, Hopkin SP (1997) Effects of temperature on the relative toxicities of Cd, Cu, Pb, and Zn to *Folsomia candida* (Collembola). *Ecotoxicol Environ Saf* 37:125–130
- Scicchitano DA, Pegg AE (1987) Inhibition of O6-alkylguanine-DNA-alkyltransferase by metals. *Mutat Res* 192:207–210
- Smit CE, van Gestel CAM (1998) Effects of soil type, prepercolation, and ageing on bioaccumulation and toxicity of zinc for the springtail *Folsomia candida*. *Environ Toxicol Chem* 17:1132–1141
- van Straalen NM, Schobben JH, de Goede RG (1989) Population consequences of cadmium toxicity in soil microarthropods. *Ecotoxicol Environ Saf* 17:190–204
- Wang ZL (2004) Zinc oxide nanostructures: growth, properties and applications. *J Phys Condens Matter* 16:829–858
- Xia T, Kovochich M, Brant J, Hotze M, Sempf J, Oberley T, Sioutas C, Yeh JI, Wiesner MR, Nel AE (2006) Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano Lett* 6:1794–1807
- Yang SW, Becker FF, Cn JYH (1996) Inhibition of human DNA ligase I activity by zinc and cadmium and the fidelity of ligation. *Environ Mol Mutagen* 28:19–25