



Study of zinc oxide nanoparticles and zinc chloride toxicity to annelid *Enchytraeus crypticus* in modified agar-based media

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Abstract

Acute toxicity of zinc oxide nanoparticle (ZnO-NP, mean particle size diameter of 10 nm) powder and water-soluble salt of zinc (ZnCl₂) to annelid *Enchytraeus crypticus* was tested using an agar-based nutrient-enriched medium with the addition of kaolin and humic acids (HA). Adults of the *E. crypticus* were cultivated in pure agar and in three types of modified exposure media containing different proportions of model soil constituents. Potworms were exposed to zinc in both forms (1–1000 mg kg⁻¹ of agar) for 96 h. In experiments with ZnCl₂, toxicity of zinc was the highest in pure agar followed by agar with HA and agar with kaolin and HA and the lowest toxicity was observed in agar with kaolin. The corresponding LC₅₀ values were 13.2, 28.8, 39.4, and 75.4 mg kg⁻¹ respectively. In contrast, zinc in the form of ZnO-NPs was most toxic in the presence of HA followed by pure agar, agar with kaolin, and kaolin with HA. In this case, LC₅₀ values were 15.8, 43.5, 111, and 122 mg kg⁻¹ respectively. Scanning electron microscopy revealed that the smallest agglomerates occurred in the presence of kaolin, where ZnO-NPs were sealed in a kaolin shell. This effect reduced the bioavailability and toxicity of the NPs. In contrast, larger agglomerates were observed in the presence of HA but a larger amount of zinc was dispersed in the volume of agar.

Keywords Agar-based cultivation media · Kaolin · Humic acids · Nanoparticles · Interactions · Physicochemical characteristics · Worms

Introduction

The increasing usage of engineered nanomaterials in nearly all areas of human activity is associated with the increasing uncontrolled release into the soil environment. The complex

nature of the soil, however, prevents the determination of the exact amount of NPs due to various limitations and a prediction of the impact for soil organisms in the environment (Caballero-Guzman and Nowack 2016).

Numerous studies of ZnO-NP toxicity for terrestrial organisms (Hooper et al. 2011; Khare et al. 2011; Li et al. 2011; Ma et al. 2009; Waalewijn-Kool et al. 2013; Wang et al. 2009) have been published to date. Ecotoxicity tests of nanomaterials carried out in soil matrices provide, however, variable and poorly comparable results. The sources of data variability are the highly variable soil composition and inconsistency of the processes used for characterization of NPs (Handy et al. 2012). The features of exposure media affect the behavior of nanomaterials and in particular the process of agglomeration, which is crucial for bioavailability of nanoparticles (Nel et al. 2009; Oprsal et al. 2015). The influence of agglomeration on resulting NP toxicity was evaluated in several studies (Albanese and Chan 2011; Bai et al. 2010; Darlington et al. 2009; Everett et al. 2014; Hrda et al. 2016; Zhu et al. 2009). Most of the results indicate that agglomeration leads to reduction in nanoparticle toxicity and to immobilization of particles in a soil environment.

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The study of the physical-chemical transformation processes of NPs in real soil and the description of NP interactions with inorganic and organic soil constituents should be an integral part of studies dealing with ecotoxicity of nanomaterials (Ben-Moshe et al. 2013). These processes affect the availability of NPs in porous media, transport to water systems, plant intake, etc. It has been demonstrated several times that the behavior, transport, and mobility of NPs are strongly dependent on environmental conditions. The proportion of inorganic and organic components significantly influences the bioavailability of NPs in the soil and thus toxicity (Maurer-Jones et al. 2013).

For the detection of nanoparticles in soil and the study of their behavior, the flow field flow fractionation (FFFF) and UV spectroscopy in combination with inductively coupled plasma mass spectrometry (ICP-MS) and liquid chromatography tandem mass spectrometry (LCMS/MS) are primarily used (Maurer-Jones et al. 2013; Pachapur et al. 2016). The methods require sample modification, which consists of extracting NPs from the soil matrix and then concentrating the samples. Sample preparation for analysis is one of the major sources of data variability. Due to the low concentration of NPs in soils and higher levels of detection limits of analytical methods, a large number of extraction steps are usually required, thus making the reproducibility of the results worsen further.

One of the main problems of nanomaterial testing is the preparation of the exposure medium (Li et al. 2011). It was also determined that the choice of the spiking method affects the resulting agglomeration state and ecotoxicity (Hrda et al. 2016; Hund-Rinke et al. 2012). In laboratory tests where data reproducibility is required, agglomeration complicates interpretation (Opsal et al. 2015). In order to suppress the interfering effect of agglomeration during laboratory tests and simplify the complexity of soil, there are different approaches for nanomaterial testing in the available literature. Tests were performed, for example, in solutions containing natural organic matter, in hydroponic solutions, or in the presence of certain soil components such as a sandy environment (Canas et al. 2011; Jahan et al. 2018; Li et al. 2011).

The main objective of our work was to develop a simple laboratory method enabling the study of an influence of model soil constituents on resulting nanoparticle toxicity for terrestrial organisms. Simplification of a physicochemical characterization, partial suppression of an agglomeration, and stable agglomeration status of nanoparticles during the experiment were main presumed advantages of the proposed system. Soil was replaced in the present study by an agar and the influence of the soil constituents on the behavior and resulting toxicity of zinc oxide nanoparticles was studied. Agar medium was modified with the addition of humic acids, kaolin, and their combination. Given that most of the studies suggest that toxicity of ZnO-NPs is connected more with ions released after

their dissolution rather than with the effect of the nanoparticles itself (Heggelund et al. 2014; Hrda et al. 2016; Kool et al. 2011; Kwak and An 2015), experiments with zinc oxide nanoparticles and soluble zinc chloride were performed in parallel.

Materials and methods

Chemicals

ZnO-NP powder with a purity of $\geq 99.5\%$ was purchased from Bochemie Group Bohumín, Czech Republic. The nominal range of particle diameters was 5–50 nm with an average of 10 nm. These particles were spherical with the hexagonal crystalline structure of zincite. ZnCl_2 p.a. purchased from PENTA, Czech Republic, was used in the tests evaluating the toxicity of dissociated Zn^{2+} cations. NaCl p.a. purchased from LACHEMA, Czech Republic, was used to evaluate the toxicity of chloride anions. $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ with a purity of $\geq 99\%$ obtained from Sigma-Aldrich (USA) served as a dispersant and stabilizing agent during the ZnO-NP dispersion in demineralized water.

Test species

The culture of *Enchytraeus crypticus* was maintained in plastic Petri dishes with ventilation (Fisherbrand, PS, aseptic, 90×14.2 mm, Fisher Scientific, USA) filled with the agar at a temperature of 21 ± 2 °C. Culture media was prepared using powder agar (Dr. Hoffmann, Czech Republic), distilled water, and water soluble salts (NaHCO_3 , KCl , MgSO_4 , Lach-ner, Czech Republic, and CaCl_2 , PENTA, Czech Republic.). Potworms were cultivated in the dark and fed finely ground oatmeal twice a week. Adults with a well-developed clitellum were used for the tests.

Experiments with ZnCl_2

Exposure media was prepared by the addition of dry agarose (1.5%) to ZnCl_2 solution in demineralized water. The pH of the mixtures was adjusted to 7 with the 0.1 M phosphate buffer (LACHEMA, Czech Republic) and concentrations of the buffer in resulting solutions varied from 0.3 to 10 mM. Mixtures were then vigorously stirred with a magnetic stirrer at 95 ± 5 °C for 30 min. The tested concentrations of zinc were 1, 10, 20, 30, and 60 mg kg^{-1} of agar (wet weight). This concentration range was chosen because an LC_{50} of 37.2 mg Zn kg^{-1} in pure agar was found in our previous study (Hrda et al. 2016). The hot agarose medium was then poured into plastic Petri dishes with ventilation of both the bottom and the lid to prevent the escape of the test organism from the Zn-containing environment. To facilitate faster penetration of organisms into the medium, notches were created in the cooled

agar using a scalpel. Concentrations of Zn and dispersion homogeneity in the media were verified using inductively coupled plasma optical emission spectrometry (ICP-OES) (Integra XL, GBC, Australia). Eight samples ($5 \times 5 \times 2$ mm) of agar media were collected from different locations and depths (surface and bottom of the agar in the dish and the layer on the lid) in each tested concentration. The relative standard deviation of Zn concentration in the samples was not higher than $\pm 3\%$. Twenty adult worms, with visible eggs in the clitellum region, were placed into each Petri dish and maintained in the dark inside a climate-controlled box (21 ± 2 °C) for a period of 96 h. Three independent replicates were performed for each tested concentration level, including the control. The endpoint of the tests was the mortality defined as the mortality in the medium with a particular concentration of zinc versus the mortality in the negative control.

The effect of zinc was also tested in all three modifications of the agar medium, i.e., in the presence of 1% kaolin (Dr. Hoffmann, Czech Republic), in the presence of 0.1% humic acids originating from weathered brown coal called oxyhumolite (Humatex, Czech Republic), and in the presence of both components. Soil constituents were also added to a control group. The preparation process of the exposure media and the experimental conditions were the same as mentioned earlier with the exception of the medium with kaolin. Red food dye (Babeta, Czech Republic) was added to agar in this case to improve the visibility of white worms in the originally white environment. Negligible toxicity of the dye up to concentration 0.5% w/w was proven within series of preliminary tests. The tested concentrations of zinc were 1, 25, 50, 100, 250, 500, and 1000 mg kg⁻¹ of agar. The range of tested concentrations was broader than in the case of pure agar because we expected soil constituents could reduce the bioavailability of zinc.

Due to the potential toxicity of the chloride anion, an experiment with NaCl was also performed. The preparation of the exposure media with NaCl and the experimental conditions were the same as for the previously described test with pure agar. The concentrations of the chloride anion in the medium corresponded to the concentration of chloride anion in the test with ZnCl₂ (50, 100, 250, 500, 1000 mg Zn kg⁻¹ of agar): 54.2, 108, 271, 542, 1084 mg Cl kg⁻¹ of agar.

Characterization of ZnO nanoparticles in colloidal solutions and exposure media

The preparation of the exposure media for ZnO-NP testing was based on the spiking procedure described elsewhere (Hrda et al. 2016). Stable-enough and well-characterized colloidal solutions with appropriate concentrations of ZnO-NPs have been for 15 min dropwise added to the hot agarose. The obtained mixture was then heated to 95 ± 5 °C and this temperature was maintained for 30 min.

The hydrodynamic diameter in the stabilized colloidal solutions (~ 50 – 1000 mg kg⁻¹), prepared using sonication for 45 min with the addition of Na₄P₂O₇·10H₂O as a dispersant, was measured using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern, UK). The conditions of sonication and colloid stabilization were selected based on the previous thorough optimization.

In addition, the effect of heating on dissolution of ZnO-NPs was monitored. Primary colloids were prepared at the same concentrations as for preparation of agar media (50, 100, 250, 500, 750, 1000 mg ZnO-NP kg⁻¹ of agar) in two replicates. The colloids were then placed in an ultrasonic bath for 45 min. One replicate was then heated to 95 °C and maintained at this temperature for 15 min. Both replicates were consequently centrifuged for 2 h at 11000 rpm. The supernatants of all samples were then analyzed by ICP-OES.

Samples of the agar media with ZnO-NP concentrations of 50 and 1000 mg kg⁻¹ prepared in all four modifications were characterized by scanning electron microscopy (SEM; JSM-5500LV, JEOL, Japan) using energy dispersive X-ray micro-analyzer IXRF systems (detector GRESHAM Sirius 10). The procedure for sample preparation and analysis conditions is described in the previous study (Hrda et al. 2016).

Experiments with ZnO nanoparticles

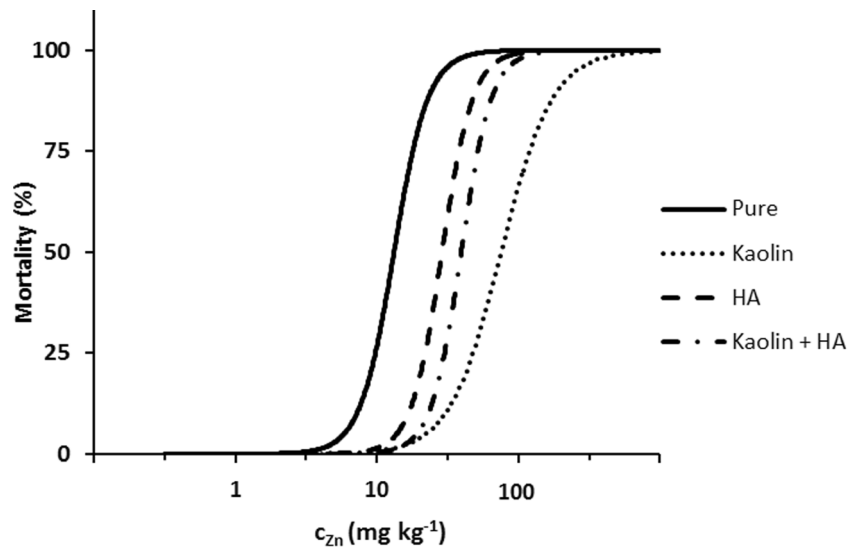
Toxicity of ZnO-NPs dispersed in pure and modified (addition of HA, kaolin, and both) agarose gels was evaluated after 96-h exposure period and mortality was selected as the test endpoint. The tested concentrations were 50, 100, 250, 500, 750, and 1000 mg ZnO-NPs kg⁻¹ of agar (40, 80, 201, 402, 603, 804 mg Zn kg⁻¹ of agar).

Results and discussion

Experiments with ZnCl₂

The dose-response curves describing acute toxicity of zinc originating from ZnCl₂ are shown in Fig. 1 (for more details, see Suppl. Mat. Fig. 5). For creating of dose-response curves and for calculation of LC₅₀ values, the module of nonlinear regression in GraphPad Prism 7 software was used. After 96 h of exposure, the worm mortality in the control group did not exceed the value of 20% in any of the experiment. Mortality in the exposure media (see Suppl. Mat. Tabs. 1–4) increased with the increasing zinc concentration in all modifications of the exposure media. The calculated values of 96-h LC₅₀ (with a corresponding 95% confidence interval) are shown in Table 1. Toxicity of zinc was the highest in pure agar (13.2 mg kg⁻¹) followed by agar with HA (28.8 mg kg⁻¹) and agar with kaolin and HA (39.4 mg kg⁻¹) and the lowest toxicity was observed in agar with kaolin (75.4 mg kg⁻¹). The addition of even an

Fig. 1 Dose-response curves describing the acute toxicity of zinc originating from ZnCl₂ in four different exposure media. c_{Zn} expressed in 10⁻³ mg Zn kg⁻¹ of agar (curves with the original data are showed at Suppl. Mat. Fig. 5)



extremely low amount of soil components reduced the toxicity of zinc. For comparison, in our previous work (Hrda et al. 2016), where 2% Bacto-Agar was used, a LC₅₀ value 37.2 (35.5–38.8) mg Zn kg⁻¹ of pure agar was found. Higher zinc toxicity (LC₅₀ 13 mg kg⁻¹ of agar) may be caused by the use of agar from another manufacturer and due to a different concentration of agar. Phipps et al. (1995) studied the toxicity of the zinc cation for *Lumbriculus variegatus* in the natural water. The LC₅₀ value after 10-day exposure was only 3 mg l⁻¹. Posthuma et al. (1997) reported an LC₅₀ value 336 (266–425) mg Zn kg⁻¹ of modified dry OECD soil for *E. crypticus* after a 4-week exposure. Garcia-Gomez et al. (2014) observed 98% inhibition of *Eisenia fetida* cocoon production after 28-day exposure to ZnCl₂ at a concentration of 1000 mg kg⁻¹ of natural soil in which experiments were performed. The toxicity of zinc cation for soil organisms cultivated in the aquatic environment is typically higher than that in the agar medium or soil. The agar medium contains polysaccharides (agarose, agarpectin) that can interact with zinc ions and reduce their bioavailability. Soil is a far more complex medium where components and physicochemical processes affect the toxicity of chemicals. The influence of soil composition may result in major differences in LC₅₀ values. Novais

et al. (2011), for example, reported LC₅₀ to *E. albidus* 72.6 mg of Zn kg⁻¹ of artificial OECD soil. Lock and Janssen (2003), in contrast, reported LC₅₀ to *E. albidus* 603 mg of Zn kg⁻¹ of natural soil LUFA 2.2.

During experiments with high concentrations of ZnCl₂ (500 and 1000 mg Zn kg⁻¹ of agar) in modified agar, where larger volumes of buffer were used to adjust pH of the mixtures, we observed the effect of the buffer on the test endpoint. The mortality was 100% in test vessels with a concentration 250 mg Zn kg⁻¹ of agar in all three modified environments, while live worms were discovered in test vessels with higher Zn concentrations (see Suppl. Mat. Tabs. 2–4). Increase in Zn concentration from 500 to 1000 mg Zn kg⁻¹ caused a decrease in the mortality in all modified media (mortality 100 vs. 57% in agar with HA; 42 vs. 33% in agar with kaolin; and 95 vs. 82% in agar with both supplements). The mortalities observed from these concentrations levels were consequently not included in the dose-response curves and calculations of LC₅₀. To verify the assumed origin of the phenomenon, an experiment with different volumes of the buffer and the same concentration of zinc was performed. The tested concentration of zinc was 250 mg kg⁻¹ of agar. The tested volumes of phosphate buffer were 3.7, 37, 74, 111, and 148 ml l⁻¹ of exposure media. The experiment was performed with the addition of both constituents (0.1% HA and 1% kaolin) with 20 adults per test vessel. The pH in all test vessels was 6 ± 0.2. At the lowest tested volume of buffer, the mortality was 100%, survival increased with increasing volume of buffer, and at the highest tested volume mortality was only 10% (see Suppl. Mat. Fig. 7). The two highest concentrations of zinc (500 and 1000 mg Zn kg⁻¹ of agar) were also tested in all three modifications without using a buffer. The pH of agars without buffer was in the range 5–6. To exclude the effect of pH on the survival of worms, another experiment was carried out. The worms were placed for 96 h into vessels filled with agar with

Table 1 LC₅₀ values for both forms of zinc obtained in all four agar-based environments

Exposure media	LC ₅₀ (mg kg ⁻¹)	
	ZnCl ₂	ZnO-NPs
Pure agar	13.2 (12.1–14.3)	43.5 (23.8–60.1)
Agar with kaolin	75.4 (65.8–86.5)	111 (73.6–157)
Agar with HA	28.8 (27.7–30.0)	15.8 (3.3–36.9)
Agar with kaolin and HA	39.4 (36.7–42.2)	122 (80.1–171)

Table 2 Concentration of zinc in non-heated and heated supernatants

C in colloids (mg l ⁻¹)	C in non-heated supernatants (mg l ⁻¹)	C in heated supernatants (mg l ⁻¹)
111	28.5	33.0
221	28.2	29.1
553	32.0	34.8
1105	35.0	32.1
1658	26.2	27.5
2210	28.3	22.8

pH 5 and 6. The mortality in experiments with 500 and 1000 mg Zn kg⁻¹ of agar without buffer was 100% in all three modifications. In the control experiment in agar without Zn with pH 5 and 6 after 96 h, the survival was 100%.

Regarding the effect of the chloride anion (Schrader et al. 1998), a test with NaCl was performed. After 96 h of exposure, no mortality was observed in concentrations up to 1084 mg Cl kg⁻¹ of agar.

Characterization of ZnO nanoparticles in colloidal solutions and exposure media

The hydrodynamic diameter of ZnO nanoparticles in the stabilized colloidal solutions (50–1000 mg kg⁻¹), prepared using sonication for 45 min with the addition of Na₄P₂O₇·10H₂O as a dispersant, was measured using DLS. The measurement revealed a relatively narrow range of the hydrodynamic diameter of all colloids. The average diameter of nanoparticle agglomerates was 268 ± 28 nm.

With regard to the effect of heating on dissolution of ZnO-NPs, ICP-OEC analysis showed that heating did not affect the dissolution (see Table 2). The average zinc concentration in

supernatants was 29.7 ± 2.7 mg l⁻¹ in non-heated colloids and 29.9 ± 3.7 mg l⁻¹ in heated colloids. Independently on a heating status, there was no observable relationship between Zn content in primary ZnO-NP colloids and supernatants.

SEM with EDX microanalyzer was used for the characterization of nanoparticles in the exposure media. SEM images of agar samples with ZnO-NP concentrations 50 (left column) and 1000 mg kg⁻¹ (right column) are shown in Fig. 3. In the samples of pure agar, the so-called bubbles of agar were observed (Fig. 3, labeled as “1”). The analysis of the chemical composition (see Supl. Mat. Fig. 1) revealed that they did not contain detectable zinc. The agglomerates of ZnO with different lateral dimensions were detected on the composite of ZnO-agar. There were detected preferable small agglomerates (size < 1 μm labeled as “2,” upper right image) for samples with 50 mg ZnO-NPs kg⁻¹ and micrometric agglomerates (lateral size > 1 μm) for samples with 1000 mg ZnO-NPs kg⁻¹ (see particles labeled as “4” on Fig. 3). Analysis of the sample area (labeled as “3”) indicated that ZnO was dispersed throughout the entire volume of the agar in both samples. The very bright objects observed on the sample surface had different signal intensity on the SEM, which is related to the surface topology and not the change in chemical composition.

Objects with a different size were observed in agar containing 1% kaolin. These objects always showed higher intensities of silicon and aluminum lines compared to zinc (see Supl. Mat. Fig. 2), leading to the conclusion that they were not ZnO agglomerates, but especially kaolin formations. In these samples, agglomerates of ZnO were not found up to the level of the device resolution and Zn/C intensity ratio measured on the different places of the flat surface was similar. In the presence of kaolin, nanoparticles were better dispersed than in the case of pure agar.

Fig. 2 Dose-response curves describing the acute toxicity of zinc originating from ZnO-NPs in four different exposure media. c_{Zn} expressed in 10⁻³ mg Zn kg⁻¹ of agar (curves with the original data are showed at Supl. Mat. Fig. 6)

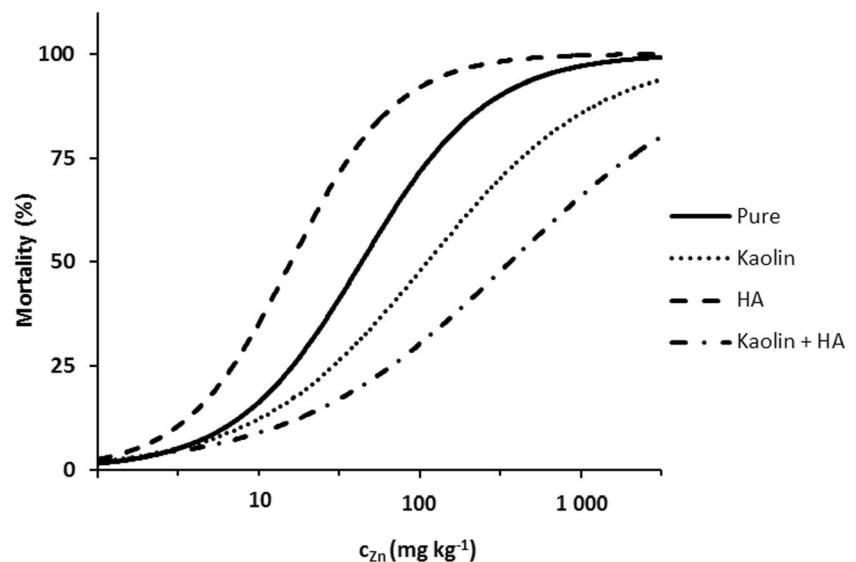
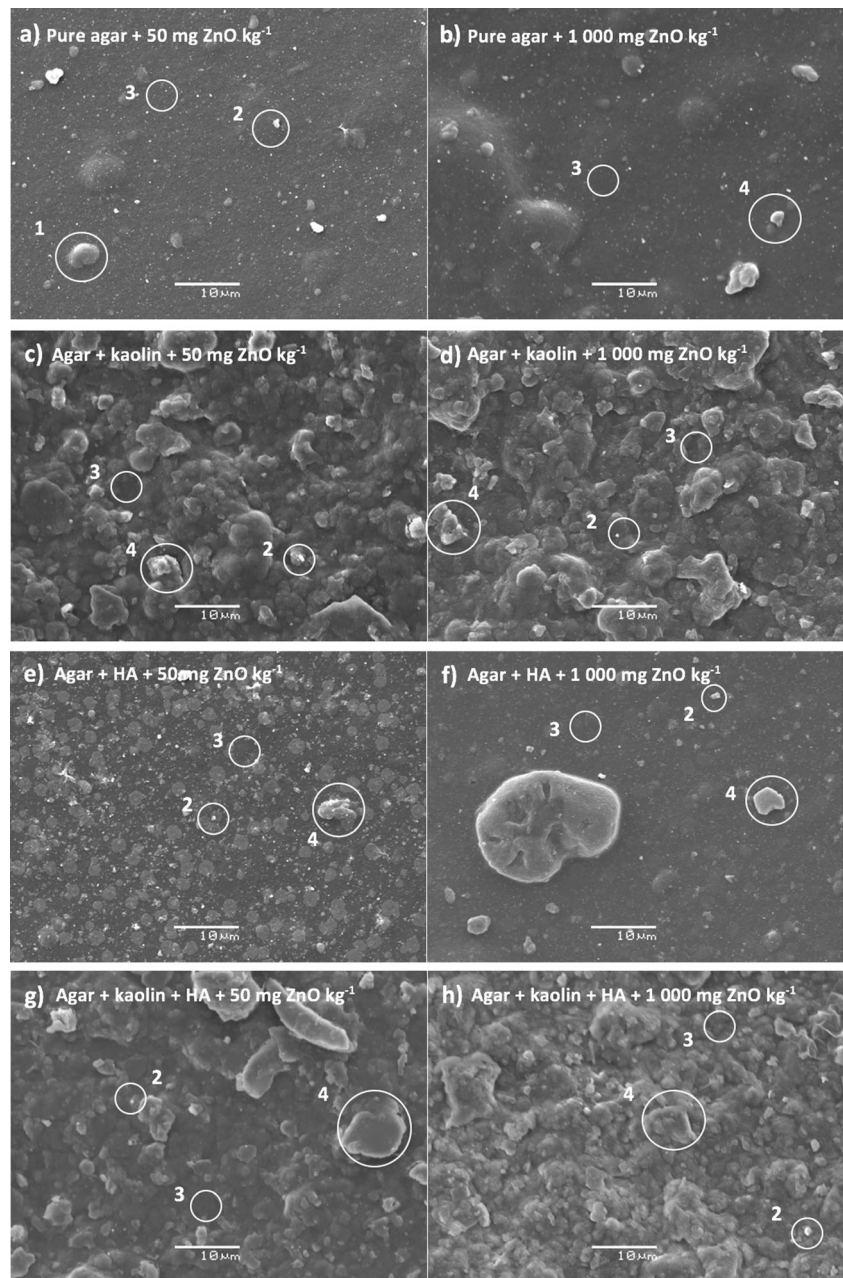


Fig. 3 a–h SEM images of agar with ZnO-NPs, $\times 2000$ magnification. Examples of the objects analyzed with EDX: (1) “bubble” of agar, (2) small particle, (3) area, (4) large particle



Nanoparticles were coated with kaolin during agar preparation, which in all probability prevented the formation of larger ZnO agglomerates.

The behavior of ZnO was completely different in the presence of 0.1% HA, in contrast to the behavior in the presence of kaolin. Larger agglomerates of ZnO ($> 1 \mu\text{m}$, labeled as “4”) were also observed in these samples, however, in a smaller amount in the same area than in pure agar. Even in this case, zinc was detected in the entire agar volume. The Zn/C intensity ratio in the flat surface (see Supl. Mat. Fig. 3) without agglomerates (labeled as “3”) was higher than that in the case of pure agar, indicating that a larger amount of zinc was dispersed in the agar.

Similar behavior of ZnO was observed in agar samples containing kaolin and HA (Fig. 3g, h) as in agar with kaolin. The EDX analysis (see Supl. Mat. Fig. 4) revealed that the observed formations (labeled as “4”) were again made of kaolin and zinc was detected in the entire volume of the agar. In this case, extremely small agglomerates of ZnO were found (labeled as “2”); however, the size and numbers of agglomerates were still lower than those in pure agar or agar with HA.

Experiments with ZnO nanoparticles

The dose-response curves describing acute toxicity of zinc originating from ZnO-NPs are shown in Fig. 2 (for more

details see Suppl. Mat. Fig. 6). After 96 h of exposure, the worm mortality in the control group did not exceed the value of 20% in any of the experiment. Mortality in the exposure media (see Suppl. Tab. 5–8) increased with the increasing zinc concentration in all modifications of the agar. The calculated values of 96-h LC₅₀ (with a corresponding 95% confidence interval) are shown in Table 1. Toxicity of zinc was the highest in agar with HA (15.8 mg kg⁻¹) followed by pure agar (43.5 mg kg⁻¹) and agar with kaolin (111 mg kg⁻¹) and the lowest toxicity was observed in agar with kaolin and HA (122 mg kg⁻¹). In comparison with the ZnCl₂ results, HA even increased the bioavailability and toxicity of zinc. The presence of kaolin, on the other hand, decreased the toxicity of zinc originating from ZnO-NPs almost ten times in comparison with the ZnCl₂ in pure agar. When HA and kaolin were combined, the effect of kaolin proved to be dominant. The dose-response curves show (see Fig. 2) that the steepest curve was obtained in the agar with HA, where the toxic effect was most potent. An almost-comparable shape of dose-response curves in agar with kaolin and agar with kaolin and HA revealed that only kaolin was able to reduce bioavailability of zinc originating from ZnO-NPs. In the presence of 5 and 40% kaolin, Owojori et al. (2009) also observed significantly different mortalities of earthworms *Eisenia fetida* exposed to 1000 mg of Zn kg⁻¹ of soil. Given that our SEM analysis revealed that more zinc was dispersed in the volume of agar in the presence of HA, it seems that HA increased the amount of dissolved zinc ions (Fig. 3). These findings are consistent with other studies where the ability of HA to increase dissolution of zinc or other metals was shown (Shao-Wei et al. 2011; Sanchez-Marin et al. 2007) Observed mortality was thereby induced by well-dispersed nanoparticles and dissolved zinc ions. For comparison, Canas et al. (2011) studied the acute and chronic toxicity of ZnO-NPs (40–100 nm) on a filter paper, in the sandy exposure medium, and in artificial soil for earthworm *Eisenia fetida*. The highest acute toxicity was observed in nanoparticles on the filter paper, significantly lower in the sandy environment (10% mortality at concentrations 10 and 100 mg kg⁻¹ and only 20% mortality at concentration 10,000 mg kg⁻¹) and only reproductive toxicity in the artificial soil. Khare et al. (2011), in contrast, reported LC₅₀s of ZnO-NPs (< 25 vs. < 100 nm) for nematode *Caenorhabditis elegans* to be only 0.32 and 2.0 mg l⁻¹ respectively. The dependence of resulting toxicity on the size of particles was commented in this work. There is no secondary characterization of ZnO-NPs in the exposure medium, however, in both studies, as the authors reported only the initial size. It is apparent from our characterization data that particle size has changed during the experiment preparation. The initial size was 10 nm on average, in colloidal solutions particles had more than 200 nm, and in exposure media there agglomerates with a different size up to more than 1 μm were present. There is a clear trend that with the increasing complexity of

the system, the toxicity of nanoparticles decreased. This is apparent because the size changed during interactions with components of the concrete medium. The different sizes of the organism also play a role in interactions with different sized nanoparticle agglomerates.

Conclusions

The proposed agar-based experimental system was used to study the influence of model soil constituents (kaolin and humic acids) on the resulting toxicity of ZnO-NPs. In the agar medium, we easily demonstrated that the kaolin was able to reduce bioavailability of zinc originating from both forms while HA had an opposite effect depending on the zinc form. The use of agar provides an advantage particularly in terms of simple and quick preparation of the laboratory experiment and easy preparation of the samples for the secondary characterization of nanoparticles in exposure media. Partial suppression of the agglomeration, stable agglomeration status of nanoparticles during the whole experiment, and almost-uniform distribution of the nanoparticles in the entire volume of exposure media enable the study of the effects of nanoparticles under stable and well-adjustable conditions. Highly artificial character of the proposed agar system, which is connected with reduced degree of its environmental relevance, obviously prevents the use of this method as a substitute of assays performed in a real soil matrix. On the other hand, application of the proposed cheap and simple approach as a tool in a first tier of environmental risk assessment or for studies dealing with an influence of physicochemical conditions on the nanoparticle toxicity could be beneficial.

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