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ORIGINAL PAPER

Toxicity of zinc oxide nanoparticles to the annelid *Enchytraeus crypticus* in agar-based exposure media

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Toxicity of zinc oxide nanoparticle (ZnO-NPs) powder and water soluble salt of Zn ($ZnCl_2$) to the annelid *Enchytraeus crypticus* was tested in agarose gel. Influence of the spiking method on the resulting size of nanoparticles and on *E. crypticus* mortality was studied. Two methods of ZnO-NPs powder (mean particle size diameter of 10 nm) introduction into the exposure media were used. In the first method, the nano-powder was initially cryogenically ground with dry agar followed by an addition of water. The second procedure began with re-suspension of nanoparticles in demineralized water containing a dispersant (sodium pyrophosphate decahydrate). The obtained colloid was subsequently mixed with hot agar gel. Relative mortality in worms observed after 96 h of their exposure to the ZnO-NPs concentrations (all in mg of ZnO-NPs per kg of agar) of 50, 100, 200, 500 and 1000 in the cryogenically ground medium ranged between 28.9 % and 34.4 % and it did not exhibit any concentration dependence. When the second method of exposure media preparation was applied, the relative mortality ranged from 0 % to 66.6 % in the same concentration region depending on the concentration. Scanning electron microscopy (SEM) revealed the presence of large agglomerates (1–10 μm in diameter) in the media prepared by cryogenic grinding with the highest concentration of ZnO-NPs. Neither the cryogenically ground media with lower ZnO-NPs concentrations nor any media prepared from colloidal solutions contained agglomerates exceeding 100 nm, detectable by SEM. Hydrodynamic diameters of particles in the colloids used in the second method of agar preparation were measured using dynamic light scattering (DLS) and ranged between 164 nm and 240 nm. The observed toxicity was thus clearly dependent on the size of ZnO-NPs agglomerates and the technique of exposure media preparation. Experimentally detected LC_{50} value for dissolved Zn^{2+} was 37.2 mg kg^{-1} in agar. The same concentration of Zn induced an approximately 30 % mortality of *E. crypticus* when administered in form of cryogenically ground ZnO-NPs with agar. No observable effects were found at this ZnO-NPs concentration when the exposure medium was prepared from the colloid solution.

Keywords: terrestrial ecotoxicity test, zinc oxide nanoparticles, agar, potworm, *Enchytraeus crypticus*

Introduction

Nanoparticles of zinc oxide (ZnO-NPs) exhibit unique optoelectronic, catalytic and antimicrobial properties, which make them attractive for a wide range of industrial applications. The global annual production of ZnO-based nanomaterials was estimated to be 550 t in 2010 with the European share be-

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ing approximately 55 t (Piccinno et al., 2012). A more recent estimate of the European production was carried out using probabilistic modeling. Approximately 1600 t of ZnO in the form of nanomaterials was reportedly produced in the European Union (EU) in 2012 (Sun et al., 2014). Expanding production and use of ZnO-NPs naturally increase the potential of their release into the environment. Modeled ZnO-NPs concentrations in the EU are in orders of 10^{-1} μg kg^{-1} in soils and of 10^{-2} μg L^{-1} in surface watersand in the units of μg kg^{-1} in sediments. Estimated effluents from European sewage treatment plants (STP) are in orders of 10^{-1}μg L^{-1} and in tens of μg kg^{-1} for sludge (Gottschalk et al., 2009). It can be reasonably assumed that a gradual increase of ZnO-NPs applications will cause further increase of their concentration in various components of the environment. Since it is extremely difficult to reliably predict the future trend of this increase, it is virtually impossible to correctly define environmentally relevant concentrations for the ecotoxicity tests performed as an integral part of the risk assessment procedure (Bondarenko et al., 2013).

Photocatalytic activity, release of ionic Zn^{2+}, and particle-induced generation of reactive oxygen species (ROS) rank among the main contemplated mechanisms of the ZnO-NPs toxicity (Ma et al., 2013). Exposure to ZnO nanoparticles may cause inflammatory response (Giovanni et al., 2015) and cytotoxicity (Yin & Casey, 2015). Several studies have demonstrated that ZnO-NPs may contribute to DNA damage, including clastogenicity (Dufour et al., 2006) or phototoxicity under UV exposure (Yin & Casey, 2015). The contribution of particular mechanisms to the overall toxicity depends on the nanoparticle features (surface coating, particle size, purity, solubility, etc.), environment characteristics (pH, temperature, dissolved organic matter, etc.) and on the exposed organism. However, thus far, there is no sufficient amount of studies on specific test species or specific physico–chemical parameters that would allow for statistical analysis of correlation between the physico–chemical properties and ecotoxicity. Numerous ecotoxicological studies on ZnO-NPs toxicity for terrestrial organisms have been conducted on nematodes and earthworms (Hu et al., 2010; Khare et al., 2011; Li et al., 2011; Ma et al., 2009, 2011; Wang et al., 2009). The authors reported variable toxicity data due to different properties of the used nanoparticles, the used test organisms and their life stage and due to different test procedures. These studies suggest, however, quite high acute toxicity of ZnO-NPs. Results of toxicity and ecotoxicity studies tend to be significantly affected by the behavior of nanoparticles in physiological fluids or environmental media. Agglomeration and aggregation of nanoparticles are considered phenomena having a fundamental impact on processes occurring at the nano-bio interface (Nel et al., 2009). The role of nanoparticles agglomeration was evaluated within several cytotoxicity and ecotoxicity studies (Bai et al., 2010; Everett et al., 2014; Limbach et al., 2005; Wu et al., 2010; Zhang et al., 2007; Zhu et al., 2009).

The main objective of our work was to develop a simple routine method allowing for the removal of errors in ecotoxicological tests associated with the agglomeration of nanoparticles in the test medium. We wanted to demonstrate the effect of different ways of nanoparticles introduction into the exposure medium on their final size. We tried to verify the suitability of agar as the exposure medium in terrestrial ecotoxicological tests. The applied experimental design was inspired by the work published by Li et al. (2011), while annelid *Enchytraeus crypticus* was used as the model species instead of *Eisenia fetida* to extend the possible test battery for the ecotoxicological testing in agar-based media. *Enchytraeids* are ecologically relevant soil organisms. The enchytraeid reproduction test guidelines (ISO, 2004; OECD, 2004) recommend using the species *E. albidus*, although a suggested alternative is *E. crypticus* with shorter generation time and the advantage of easy cultivation in agar.

The testing media preparation described by Li et al. (2011) was based on sonication of ZnO-NPs powder mixed with hot agar. Although the particle size of primary colloid declared by the manufacturer was (30 ± 5) nm, agglomerates with the size of several micrometers were visible on the transmission electron microscope (TEM) images of the resulting media. Our intention was to find means of media preparation enabling to preserve the nanomaterial character of the tested particles more efficiently. Two methods of ZnO-NPs introduction into agar were suggested. The results of ZnO-NPs toxicity tests performed with both above-mentioned media were compared with the results of tests with a water soluble salt of Zn.

Theoretical

The dose-response curve was obtained by fitting of experimental data using the Boltzmann model:

$$y = y_{\min} + (y_{\max} - y_{\min})/(1 + \exp((P_1 - x)/P_2)) \quad (1)$$

where y is the relative mortality (%), x is ln of the Zn concentration in agar (mg of Zn per kg of agar), P_1 is the inflection point corresponding to LC_{50}, and P_2 is the slope. The curve was characterized by high regression rabat (RR = 99.42 %) and steep slope (P_2 = 0.05 ± 0.025).

Experimental

Chemicals and test species

ZnO-NPs powder with the purity of 99.5 % was purchased from Bochemie Group Bohumín, Czech Republic. The nominal range of particle diameters as pro-

vided by the manufacturer was 5–50 nm with the 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 Cl^- anions. $Na_4P_2O_7 \cdot 10H_2O$ with the purity of ≥ 99 % obtained from Sigma–Aldrich (USA) served as a dispersant and stabilizing agent during the ZnO-NPs dispersion in demineralized water. Culture media were prepared using high purity Bacto-Agar from BD, USA. Experimental organisms *E. crypticus* were kindly provided by the Research Centre for Toxic Compounds in the Environment (RECETOX), Czech Republic. The culture of *E. crypticus* was maintained in the agar at the temperature of (20 ± 2) ℃. Animals were fed by finely ground oatmeal twice a week. Adults with a well-developed clitellum were used for the tests.

Experimental design

Agarose gel was selected as the testing medium as it is supposed to suppress nanoparticle agglomeration and mitigate negative effects of this process on the ecotoxicity test results. To be able to distinguish between the ZnO-NPs nanoparticle effect and the effect of released Zn^{2+} ions, a test with water soluble salt $ZnCl_2$ was first performed. Due to the potential toxicity of the chloride anion, also an experiment with NaCl was performed.

Exposure of E. crypticus to $ZnCl_2$ and NaCl

Exposure media were prepared by adding 1 g of dry agarose to 49 mL of $ZnCl_2$ solution in demineralized water (one test vessel) and under subsequent vigorous stirring of the mixture with a magnetic stirrer at (85 ± 5) ℃ for 1 h. A preliminary test was conducted with a wide range of tested concentrations (all in mg of ZnO-NPs per kg of agar wet mass): 0, 50, 100, 200, 500 and 1000. Concentrations of $ZnCl_2$ in water solutions were selected so that the content of Zn in the media matched the content of Zn in the tests with ZnO-NPs. The hot agarose medium was then poured into plastic Petri dishes (90 × 14 mm) with ventilation of both the bottom and the lid to prevent the escape of the test organism from the Zn containing environment. To facilitate penetration/drilling of organisms into the medium, notches were created in the cooled agar using a scalpel. Concentrations of Zn and dispersion homogeneity in the resulting media were verified using optical emission spectrometry with inductively coupled plasma (ICP-OES) (Integra XL, GBC, Australia). Fifteen samples (5 × 5 × 2 mm) 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 ± 3 %.

Ten adult worms, with visible eggs in the clitellum region, were placed into each Petri dish and maintained in the dark inside a climate controlled (20 ℃) box for the period of 96 h. For each tested concentration level, including the control, three independent replicates were performed. The end-point of the tests was the relative mortality defined as the mortality in the medium with a particular concentration of Zn^{2+} versus the mortality in the negative control (pure agar). After the preliminary test (0–1000 mg kg^{-1} agar), in which the mortality of 100 % was observed at 100 mg of Zn^{2+} per 1 kg of agar, concentrations (all in mg of Zn^{2+} per kg of agar) of 0, 2, 4, 6, 10, 20, 40 and 60 were tested.

Preparation of exposure media with NaCl and also the experimental conditions were the same as for the previously described test with $ZnCl_2$. Concentrations of the chloride anion in the medium corresponded to the concentration of Cl^- in the preliminary test with $ZnCl_2$ (all in mg Cl^- per kg of agar): 43.56, 87.12, 174.28, 435.60, 871.20.

Exposure of E. crypticus to ZnO-NPs

Cryogenic grinding of dry agar with ZnO-NPs powder and dispersion of the obtained mixture in water were the key steps of the first method of nanoparticle insertion into the agarose gel. A polycarbonate grinding vial of a cryogenic mill (6970EFM Freezer/Mill, SPEX SamplePrep) was loaded with 1 g of dry agarose and the appropriate amount of ZnO-NPs powder. The following operational conditions in the cryogenic mill were applied: precooling time of 10 min; grinding time of 1 min; recooling time of 1 min; three working cycles; impactor frequency of 12 Hz. The obtained powder was transferred into an Erlenmeyer flask followed by an addition of 49 mL of demineralized water. The mixture was then continuously stirred at (85 ± 5) ℃ for 1 hour. Model organisms of *E. crypticus* were exposed to the ZnO-NPs concentration (all in mg of ZnO-NPs per kg of agar) of 0, 50, 100, 200, 500 and 1000 for 96 h. The remaining experimental conditions were the same as for the previously described test with $ZnCl_2$.

The second method of experimental medium preparation was based on a stabilized colloidal solution of nanoparticles in demineralized water in the presence of a dispersant. Ultrasonically assisted suspension of ZnO-NPs powder in demineralized water proved to be inefficient; therefore, a dispersion agent was applied. After optimization of individual steps, the following procedure was thus adopted: 4 mg of the dispersant ($Na_4P_2O_7 \cdot 10H_2O$) were dissolved in 20 mL of demineralized water, the appropriate amount of ZnO-NPs was added and the obtained suspension was sonicated (Bandelin, Sonorex) for 45 min. Then, 6 mg of the dispersant were added to 1 g of dry agarose

and the obtained mixture was stirred in 29 mL of water and heated to (85 ± 5)℃. This temperature was maintained for 45 min and the ZnO colloidal solution was then added dropwise for 15 min. Exposure of the model organisms to ZnO-NPs was carried out as described in the preceding paragraph with the only difference being that the experimental medium for the negative control contained the corresponding concentration of the dispersant.

Characterization of ZnO-NPs in colloidal solution and exposure media

Samples of the agar media with concentration of (mg of ZnO-NPs per kg of agar): 50 and 1000 prepared by both procedures were characterized by scanning electron microscopy (SEM; JSM-5500LV, JEOL, Japan) using energy dispersive X-ray microanalyzer IXRF systems (detector GRESHAM Sirius 10). Within the process of sample preparation, the hot agarose gel was poured between two glass slides and cooled. The top slide was then carefully removed and the agar layer with the thickness of 3 mm was lyophilized at a temperature below –50℃ and pressure below 60 Pa for 6 h. The gilded samples were subsequently scanned under the accelerating voltage of 10 kV.

Particle size distribution in the stabilized colloidal solutions with the concentration of (mg of ZnO-NPs per kg of agar): 50 and 1000, prepared using sonication for 45 min with the addition of $Na_4P_2O_7 \cdot 10H_2O$ as a dispersant was measured using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern, UK).

In order to determine the stabilization effect on the amount of the released zinc cation, stabilized and non-stabilized series with tested concentrations of ZnO-NPs (50–1000 mg kg^{-1}) were prepared. The amount of dissolved Zn^{2+} ions in the non-stabilized and stabilized ZnO-NPs colloids was examined 10 min and 96 h after the sonication. Then, 20 mL of the colloidal solution were centrifuged for 120 min at 11000*g* and the supernatant was consequently analyzed using ICP-OES.

Results and discussion

Exposure of E. crypticus to $ZnCl_2$ and NaCl

The dose-response curve describing acute toxicity of Zn^{2+} ions originating from $ZnCl_2$ is shown in Fig. 1. After 96 h of exposure in pure agar, the worm mortality in the control group did not exceed the value of 20 %. Mortality in the exposure medium supplemented with $ZnCl_2$ increased with the increasing $ZnCl_2$ concentration. The calculated value of 96 h LC_{50} (with a corresponding 95 % confidence interval) was 37.2 (35.5–38.8) mg of Zn per kg of agar. For comparison, Lock and Janssen (2003) reported LC_{50} of Zn

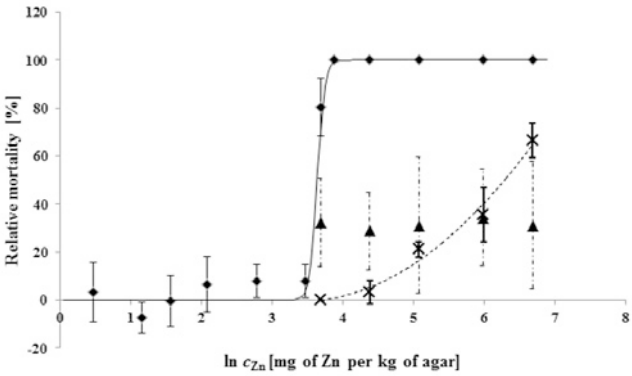

Fig. 1. Relative mortality of *E. crypticus* exposed for 96 h to $ZnCl_2$ and ZnO-NPs in agar expressed as a ratio of mortality in a particular experimental group to the negative control (pure agar). ZnO-NPs-Cryo describes the experiment, where the nanoparticles were inserted into the agar using cryogenic grinding. ZnO-NPs-Colloid denotes the experiment, where nanoparticles were introduced in form of colloidal solution; ◆ – $ZnCl_2$, – ZnO-NPs-Cryo, × – ZnO-NPs-Colloid.

to *E. albidus* to be 603 mg of Zn per kg of soil. Novais et al. (2011), in contrast, reported LC_{50} of Zn to *E. albidus* to be only 72.6 mg of Zn per kg of soil. The different values result from the different properties of the used soil. In the first case, toxicity was tested in OECD soil while in the latter case, the authors chose standard natural soil (LUFA 2.2) because it promotes the bioavailability of metals. The differences indicate that soil properties and physico–chemical processes in soil significantly influence the toxicity of zinc.

Regarding the effect of the chloride anion (Schrader et al., 1998), a test with NaCl was performed. After 96 h of exposure in agar, mortality was not observed in any of the tested concentrations. Also, no reproduction effect was observed up to the concentration of 1380 mg Cl^- kg^{-1} of soil for *E. crypticus* and the EC_{50} for reproduction was 2270 mg of Cl per kg of soil (Posthuma et al., 1997). The highest tested concentration in this study was approximately 870 mg of Cl per kg of agar, which is far below the expected threshold effect.

The ability of agar-based media to ensure fairly close contact of the experimental organism with the tested chemical substance without interferences decelerating its absorption into the body can explain the shape of the dose-response curve (Fig. 1). Oral uptake is also an important route of exposure because *E. crypticus* feeds on the agar during the test.

Characterization of ZnO-NPs in exposure media

SEM images of samples with concentrations (mg of ZnO-NPs per kg of agar) of 1000 and 50 prepared by cryogenic grinding are shown in Figs. 2a and 2b.

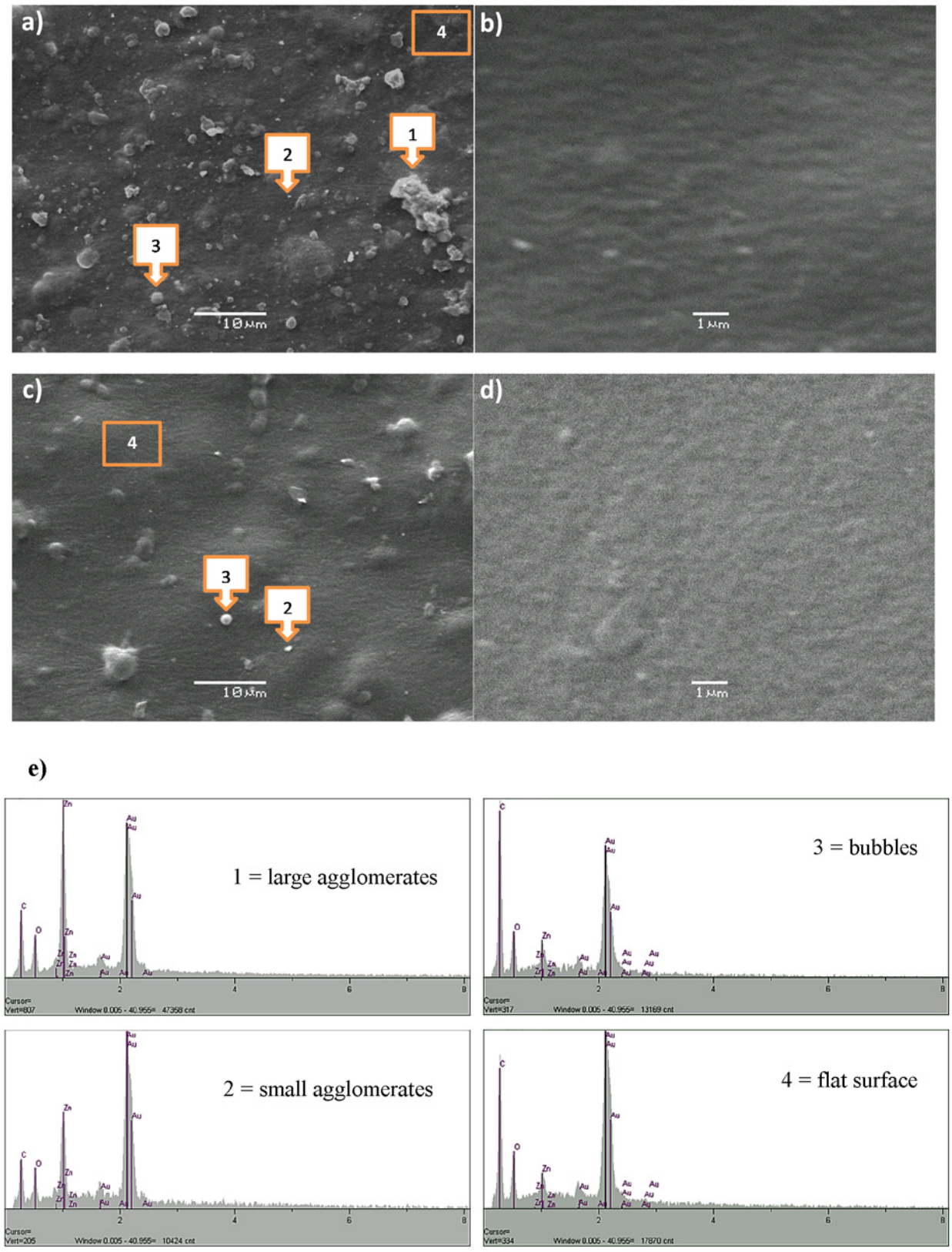

Fig. 2. Objects observed using SEM in exposure media with (mg of ZnO-NPs per kg of agar): 1000 (a, c) and 50 (b, d) prepared by procedure 1 (a, b) and procedure 2 (c, d) – large (1) and small (2) agglomerates; bubbles (3) and flat surface (4) of agar and ED XRF spectra (e) corresponding to locations highlighted in image (a); signal of gold (Au) visible on the spectra originates from sample gilding.

Energy dispersive X-ray fluorescence (ED XRF) spectra, showing the chemical composition of particular objects on SEM images, are presented in Fig. 2e.

Four types of objects with different intensities of Zn and C analytical lines occurring in the corresponding ED XRF spectra (Fig. 2e) can be discovered in Fig. 2a. Although objects (1) and (2) significantly vary in size, the Zn/C intensity ratio is approximately 3 for both of them. This indicates that these objects contain high concentrations of Zn and were therefore described as large and small agglomerates of ZnO-NPs. The Zn/C intensity ratio for objects (3) and (4) was around 1/3, which indicates the dilution of ZnO-NPs with the carbon-containing medium. These objects were very likely bubbles (3) and flat surface (4) of agar with dispersed ZnO nanoparticles or Zn^{2+} ions.

As it can be seen in Figs. 2a and 2e, a significant portion of ZnO-NPs in the more concentrated sample was present in form of large agglomerates (1–10 μm) although particles with the size below the equipment resolution ($\leq$ 100 nm) or maybe even dissolved Zn^{2+} ions were also present. The exposure medium with ZnO-NPs concentration of 50 mg of ZnO-NPs per kg of agar (Figs. 2b and 2d) did not contain any large agglomerates and the entire dose of ZnO-NPs was apparently in form of dispersed nanoparticles or dissolved ions.

SEM images of samples with concentrations (mg of ZnO-NPs per kg of agar) of 1000 and 50 prepared from the colloidal solution are shown in Figs. 2c and 2d. The SEM image for 1000 mg of ZnO-NPs per kg of agar (Fig. 2c) only contains small agglomerates and no visible Zn containing particles can be observed on the SEM image for 50 mg of ZnO-NPs per kg of agar (Fig. 2d).

Characterization of ZnO-NPs in colloidal solution

Results of particle size distribution evaluation in colloidal solutions presented in Fig. 3 indicate a relatively narrow particle size range for both measured concentrations while the mean hydrodynamic diameters were 164 nm and 240 nm for colloids with concentrations (mg of ZnO-NPs per kg of agar) of 50 and 1000, respectively.

The amount of dissolved Zn^{2+} ions in the non-stabilized and stabilized ZnO-NPs colloids was examined 10 min and 96 h after the sonication (Fig. 4.). Concentrations of Zn in the non-stabilized samples analyzed 10 min after the preparation ranged between 2.2 and 8.1 mg L^{-1} and after 96 h between 2.9 mg L^{-1} and 6.8 mg L^{-1}. The Zn concentration increased with the increasing ZnO-NPs concentration. Zn concentrations in the stabilized samples analyzed 10 min after the colloid preparation ranged between 17.7 mg L^{-1} and 20.3 mg L^{-1}, which does not correlate with the total amount of ZnO-NPs in the colloids (50–1000 mg L^{-1}). Similar concentrations in the range from 15.3 mg L^{-1} and 19.2 mg L^{-1} were measured also after 96 h. These results indicate that dissolution

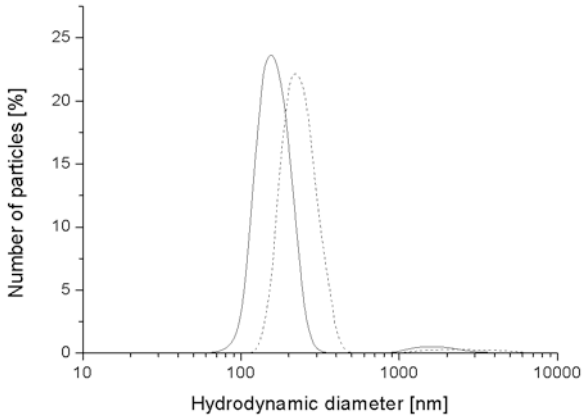

Fig. 3. Particle size distribution of colloidal solutions with concentration (mg of ZnO-NPs per kg of agar) of 50 (solid line) and 1000 (dashed line) prepared using sonication for 45 min with the addition of $Na_4P_2O_7 \cdot 10H_2O$ as a dispersant.

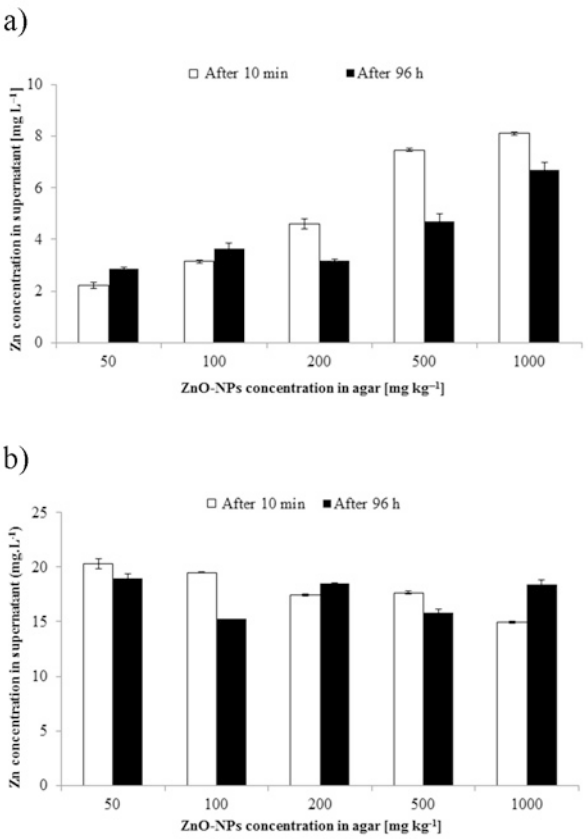

Fig. 4. Concentration of released zinc ions from non-stabilized (a) and stabilized (b) ZnO-NPs measured after 10 min and 96 h in the supernatant; bars represent the standard deviation of the average value from 3 experiments.

is apparently particle size dependent (Franklin et al., 2007). A higher amount of ions was released from stabilized particles with a smaller size and at the lowest tested concentration. Agglomeration of non-stabilized nanoparticles decreased the dissolution, which is in line with the findings of Li et al. (2011).

Based on the above SEM and DLS data it was estimated that most Zn was present in form of agglomerates smaller than 500 nm in the medium with the ZnO-NPs concentration of 1000 mg per kg of agar and in form of agglomerates smaller than 200 nm in the medium with the ZnO-NPs concentration of 50 mg per kg of agar.

Exposure of E. crypticus to ZnO-NPs

Dependence of the *E. crypticus* mortality on the ZnO-NPs dose for experiments, where nanoparticles were introduced into the agar using cryogenic grinding and pre-prepared colloidal solutions, is shown in Fig. 1. Relative mortality obtained for cryogenically treated agar ranged between 28.9 % and 34.4 % and exhibited a low reproducibility (wide error bars). Data describing the test end-point showed no dependence on the ZnO-NPs dose. The observed mortality was probably induced by a factor which remained more or less constant within the entire concentration range tested or by a factor whose effect decreased with the increasing concentration of ZnO-NPs. The content of large agglomerates increased with the increasing ZnO-NPs concentration for this method of the exposure medium preparation. As found by measuring the amount of released zinc cations from non-stabilized nanoparticles, maximum amount was 8 mg of Zn^{2+} per L in the highest tested concentration. Thus the expected concentrations of dissolved Zn^{2+} cations in agar were so low that they could not induce the observed effect. Li et al. (2011) found that the highest concentration of zinc released from ZnO-NP in agar was only 1.4 mg L^{-1}. The recorded mortality was thereby probably induced by smaller agglomerates and well dispersed nanoparticles.

The second way of exposure media preparation based on colloidal solutions led to a narrower particle size distribution with a substantially lower mean size of particles. This fact was reflected in a monotonically increasing relationship between the applied ZnO-NPs dose and the observed mortality, which ranged from 0 % to 66.7 %. In this case, the observed mortality primarily resulted from the intake by ingestion of small particles instead of dissolved Zn^{2+} ions. This is in conformity with the findings of Li et al. (2011). The concentrations of dissolved Zn^{2+} ions originating from colloids showed a very narrow distribution range in the entire region of tested concentrations (50–1000 mg of ZnO-NPs per kg of agar). The amount of dissolved Zn^{2+} ions was below the expected threshold, which it resulted from the experiment described in the section "Exposure of *E. crypticus* to $ZnCl_2$". However, the amount of Zn dissolved during the heating of agar remains questionable.

In our case, the spiking procedure significantly influenced the resulting size and ecotoxicity of ZnO-NPs. The results presented in the study of Waalewijn-Kool et al. (2012), in contrast, demonstrated that the spiking method did not influence the distribution of ZnO-NPs in soil and also that the ecotoxicity of nanoparticles to collembola *Folsomia candida* was size-independent.

Toxicity of Zn administered in form of ZnO-NPs was found to be substantially lower than the toxicity of this element administered in form of water soluble salt. Toxicity of zinc salt to collembolan *Folsomia candida* in artificial soil (Kool et al., 2011) and to earthworm *Eisenia fetida* in natural soil (García-Gómez et al., 2014) was higher than that of ZnO-NPs.

Conclusions

Agar was used as the exposure medium for testing of the ZnO-NPs toxicity to the annelid *E. crypticus*. Crucial influence of the method used for nano-powder introduction into the agar on the resulting particle size distribution and consequently on the test results was demonstrated. The amount of zinc cations released from ZnO-NPs during the dispersion of particles in the primary colloid used for agar preparation was determined. However, to elucidate the contribution of Zn^{2+} to the overall toxicity to the test organism, an analytical technique for quantitative determination of Zn^{2+} in agar would be required. The results obtained by SEM and DLS nevertheless allowed for a reliable estimate of the particle size distribution and the particle types present in the exposure media. These measurements demonstrated the crucial role of small agglomerates and well dispersed ZnO nanoparticles in the overall toxic effect. The present study is the first steps in the investigation of the ecotoxicity of NPs to soil organisms in agar-based media. The use of agar instead of soil or sediment provides an advantage particularly in terms of the nanoparticle analysis/characterization in exposure media, reduction of agglomeration and exclusion of the interactions with soil constituents in order to focus on the effects of nanoparticles.

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References

Bai, W., Zhang, Z., Tian, W., He, X., Ma, Y., Zhao, Y., & Chai, Z. (2010). Toxicity of zinc oxide nanoparticles to zebrafish embryo: a physicochemical study of toxicity mecha-

nism. *Journal of Nanoparticle Research*, *12*, 1645–1654. DOI: 10.1007/s11051-009-9740-9.

Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M., & Kahru, A. (2013). Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. *Archives of Toxicology*, *87*, 1181–1200. DOI: 10.1007/s00204-013-1079-4.

Dufour, E. K., Kumaravel, T., Nohynek, G. J., Kirland, D., & Toutain, H. (2006). Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: Genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *607*, 215–224. DOI: 10.1016/j.mrgentox.2006.04.015.

Everett, W. N., Chern, C., Sun, D., McMahon, R. E., Zhang, X., Chen, W. J. A., Hahn, M. S., & Sue, H. J. (2014). Phosphate-enhanced cytotoxicity of zinc oxide nanoparticles and agglomerates. *Toxicology Letters*, *225*, 177–184. DOI: 10.1016/j.toxlet.2013.12.005.

Franklin, N. M., Rogers, N. J., Apte, S. C., Batley, G. E., Gadd, G. E., & Casey, P. S. (2007). Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchneriella subcapitata*): the importance of particle solubility. *Environmental Science & Technology*, *41*, 8484–8490. DOI: 10.1021/es071445r.

García-Gómez, C., Babin, M., Obrador, A., Álvarez, J. M., & Fernández, M. D. (2014). Toxicity of ZnO nanoparticles, ZnO bulk, and $ZnCl_2$ on earthworms in a spiked natural soil and toxicological effects of leachates on aquatic organisms. *Archives of Environmental Contamination and Toxicology*, *67*, 465–473. DOI: 10.1007/s00244-014-0025-7.

Giovanni, M., Yue, J., Zhang, L., Xie, J., Ong, C. N., & Leong, D. T. (2015). Pro-inflammatory responses of RAW264.7 macrophages when treated with ultralow concentrations of silver, titanium dioxide, and zinc oxide nanoparticles. *Journal of Hazardous Materials*, *297*, 146–152. DOI: 10.1016/j.jhazmat.2015.04.081.

Gottschalk, F., Sonderer, T., Scholz, R. W., & Nowack, B. (2009). Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science & Technology*, *43*, 9216–9222. DOI: 10.1021/es9015553.

Hu, C. W., Li, M., Cui, Y. B., Li, D. S., Chen, J., & Yang, L. Y. (2010). Toxicological effects of TiO_2 and ZnO nanoparticles in soil on earthworm *Eisenia fetida*. *Soil Biology and Biochemistry*, *42*, 586–591. DOI: 10.1016/j.soilbio.2009.12.007.

ISO (2004). ISO 16387: Soil quality — effects of pollutants on Enchytraeidae (*Enchytraeus* sp.) – determination of effects on reproduction and survival. Geneva, Switzerland: International Organization for Standardization.

Khare, P., Sonane, M., Pandey, R., Ali, S., Gupta, K. C., & Satish, A. (2011). Adverse effects of TiO_2 and ZnO nanoparticles in soil nematode, *Caenorhabditis elegans*. *Journal of Biomedical Nanotechnology*, *7*, 116–117. DOI: 10.1166/jbn.2011.1229.

Kool, P. L., Ortiz, M. D., & van Gestel, C. A. M. (2011). Chronic toxicity of ZnO nanoparticles, non-nano ZnO and $ZnCl_2$ to *Folsomia candida* (Collembola) in relation to bioavailability in soil. *Environmental Pollution*, *159*, 2713–2719. DOI: 10.1016/j.envpol.2011.05.021.

Li, L. Z., Zhou, D. M., Peijnenburg, W. J. G. M., van Gestel, C. A. M., Jin, S. Y., Wang, Y. J., & Wang, P. (2011). Toxicity of zinc oxide nanoparticles in the earthworm *Eisenia fetida* and subcellular fractionation of Zn. *Environment International*, *37*, 1098–1104. DOI: 10.1016/j.envint.2011.01.008.

Limbach, L. K., Li, Y. C., Grass, R. N., Brunner, T. J., Hinterrmann, M. A., Muller, M., Gunther, D., & Stark, W. J. (2005). Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration and diffusion at low concentrations. *Environmental Science & Technology*, *39*, 9370–9376. DOI: 10.1021/es051043o.

Lock, K., & Janssen, C. R. (2003). Comparative toxicity of a zinc salt, zinc powder and zinc oxide to *Eiseniafetida*, *Enchytraeusalbidus* and *Folsomia candida*. *Chemosphere*, *53*, 851–856. DOI: 10.1016/s0045-6535(03)00593-9.

Ma, H., Bertsch, P. M., Glenn, T. C., Kabengi, N. J., & Williams, P. L. (2009). Toxicity of manufactured zinc oxide nanoparticles in the nematode *Caenorhabditis elegans*. *Environmental Toxicology and Chemistry*, *28*, 1324–1330. DOI: 10.1897/08-262.1.

Ma, H., Kabengi, N. J., Bertsch, P. M., Unrine, J. M., Glenn, T. C., &Williams, P. L. (2011). Comparative phototoxicity of nanoparticulate and bulk ZnO to a free-living nematode *Caenorhabditis elegans*: the importance of illumination mode and primary particle size. *Environmental Pollution*, *159*, 1473–1480. DOI: 10.1016/j.envpol.2011.03.013.

Ma, H., Williams, P. L., & Diamond, S. A. (2013). Ecotoxicity of manufactured ZnO nanoparticles – A review. *Environmental Pollution*, *172*, 76–85. DOI: 10.1016/j.envpol.2012.08.011.

Nel, A. E., Mädler, L., Velegol, D., Xia, T., Hoek, E. M. V., Somasundaran, P., Klaessig, F., Castranova, V., & Thompson, M. (2009). Understanding biophysicochemical interactions at the nano-bio interface. *Nature Materials*, *8*, 543–557. DOI: 10.1038/nmat2442.

Novais, S. C., Gomes, S. I. L., Gravato, C., Guilhermino, L., De Coen, W., Soares, A. M. V. M., & Amorim, M. J. B. (2011). Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. *Environmental Pollution*, *159*, 1836–1843. DOI: 10.1016/j.envpol.2011.03.031.

OECD (2004). OECD 220: Guidelines for testing of chemicals – Enchytraeid reproduction test. Paris, France: Organization for Economic Cooperation and Development.

Piccinno, F., Gottschalk, F., Seeger, S., & Nowack, B. (2012). Industrial production quantities and uses of ten engineered nanomaterials in Europe and in the world. *Journal of Nanoparticle Research*, *14*, 1109–1120. DOI: 10.1007/s11051-012-1109-9.

Posthuma, L., Baerselman, R., van Veen, R. P. M., & Dirven-Van Breemen, E. M. (1997). Single and joint toxic effects of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soils. *Ecotoxicology and Environmental Safety*, *38*, 108–121. DOI: 10.1006/eesa.1997.1568.

Schrader, G., Metge, K., & Bahadir, M. (1998). Importance of salt ions in ecotoxicological tests with soil arthropods. *Applied Soil Ecology*, *7*, 189–193. DOI: 10.1016/s0929-1393(97)00035-8.

Sun, T. Y., Gottschalk, F., Hungerbühler, K., & Nowack, B. (2014). Comprehensive probabilistic modeling of environmental emissions of engineered nanomaterials. *Environmental pollution*, *185*, 69–76. DOI: 10.1016/j.envpol.2013.10.004.

Waalewijn-Kool, P. L., Díez-Ortiz, M., & van Gestel, C. A. M. (2012). Effect of different spiking procedures on the distribution and toxicity of ZnO nanoparticles in soil. *Ecotoxicology*, *21*, 1797–1804. DOI: 10.1007/s10646-012-0914-3.

Wang, H., Wick, R. L., & Xing, B. (2009). Toxicity of nanoparticulate and bulk ZnO, Al_2O_3 and TiO_2 to the nematode *Caenorhabditis elegans*. *Environmental Pollution*, *157*, 1171–1177. DOI: 10.1016/j.envpol.2008.11.004.

Wu, B., Wang, Y., Lee, Y. H., Horst, A., Wang, Z., Chen, D. R., Sureshkumar, R., & Tang, Y. J. (2010). Comparative ecotoxicities of nano-ZnO particles under aquatic and aerosol exposure modes. *Environmental Science & Technology*, *44*, 1484–1489. DOI: 10.1021/es9030497.

Yin, H., & Casey, P. S. (2015). Effects of aspect ratio (AR) and specific surface area (SSA) on cytotoxicity and phototoxicity of ZnO nanomaterials. *Chemosphere*, *124*, 116–121. DOI: 10.1016/j.chemosphere.2014.11.076.

Zhang, L., Jiang, Y., Ding, Y., Povey, M., & York, D. (2007). Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). *Journal of Nanoparticle Research*, *9*, 479–489. DOI: 10.1007/s11051-006-9150-1.

Zhu, X., Wang, J., Zhang, X., Chang, Y., & Chen, Y. (2009). The impact of ZnO nanoparticle aggregates on the embryonic development of zebrafish (Danio rerio). *Nanotechnology*, *20*, 195103. DOI: 10.1088/0957-4484/20/19/195103.