Scientific Papers of the University of Pardubice, Series A; Faculty of Chemical Technology
24 (2018) 187–196.

University
of Pardubice
Faculty
of Chemical Technology

Influence of humic acids on zinc oxide nanoparticles and zinc chloride toxicity to *Enchytraeus crypticus* studied in agar-based exposure media

Kateřina Hrdá, Eliška Konopáčová, Inka Vrzáčková, and Miloslav Pouzar*

Institute of Environmental and Chemical Engineering, The University of Pardubice, CZ−532 10 Pardubice, Czech Republic

Received: June 12, 2018; Accepted: July 11, 2018

Composition of the exposure media always affects the properties of nanomaterials. In terrestrial ecotoxicology, the complex nature of the soil highly complicates the prediction of impacts of pollutants upon the environment. In our study, less complex agar-based exposure medium had been therefore used instead a soil. An acute toxicity of zinc oxide nanoparticle (ZnO-NPs, mean particle size diameter of 10 nm) powder and of water-soluble salt of zinc ($ZnCl_2$) to annelid Enchytraeus crypticus were tested, while agar-based exposure medium was modified with two sources of humic acids (sodium humate, peat) in order to study the influence of soil component and its form on toxicity of zinc. In the case of $ZnCl_2$, an addition of humic acid always reduced the toxicity. The respective values of LC_{50} were 13.2, 28.8, and 191 mg kg^{-1} for pure agar, agar with sodium humate, and agar with peat, respectively. On the contrary, the addition of sodium humate into an agar increased toxicity of ZnO NPs and the reduction in toxicity was observed only for the addition of a peat. The corresponding LC_{50} values then were 43.5 (pure agar), 15.8 (agar with sodium humate), and 304 (agar with peat) mg kg^{-1}.

Keywords: Terrestrial ecotoxicity test; Agar, Potworm, *Enchytraeus crypticus*; Humic acids; Peat

* Corresponding author, ✉ Miloslav.Pouzar@upce.cz

Introduction

Engineered nanomaterials are currently a part of nearly all human activity. Zinc oxide nanoparticles (ZnO-NPs) are one of the most interesting metal-based nanomaterials. The area of use ranges from industrial applications to personal care products. Thus, the contact with the biota in the environment is inevitable [1,2].

Soil environment contains diverse natural organic matter (NOM), which can potentially interact with the nanoparticles. These interactions influence the transformations and speciation of the nanomaterial in the environment and thus the resulting toxicity [3–5]. Humic acids (HA) which are a major component of humus have a great ability to affect the nanoparticles fate [6,7]. Humic acids are a type of dissolved organic matter as a result of the decomposition of natural organic matter (NOM). There are various reports suggesting us that the interaction with HA can increase stability of the nanoparticles [8,9]. On the other hand, there are studies which reported that HA may also reduce the bioavailability of the nanoparticles [10,11]. The main known mechanism of the ZnO-NPs toxicity is the release of the zinc cation, Zn^{2+} [12]. Released zinc ions can bind to HA which are considered the be a dominant complexing agent [13,14]. Therefore, humic acids play an important role in bioavailability and toxicity of the metal pollutants.

Some authors have already evaluated the toxicity of ZnO-NPs in the presence of HA [15,16], concluding that physicochemical properties of nanomaterials and the respective exposure conditions may induce different biological response. However, complex nature of the soil, either natural or artificial, complicates the study of the nanomaterial fate in the exposure media. In this context, the main objective of our work was to use much simpler exposure media. In the present study, a soil was replaced by the agar. The influence of the different source of HA on the characteristics and the resulting toxicity of ZnO-NPs to annelid *Enchytraeus crypticus* were studied.

This experimental setup allowed us to stabilize the agglomeration status of NPs, having been kept uniform during the whole experiment, and in such a way to be able to improve the reproducibility of the results. The possibility of secondary characterization of NPs dispersed in cultivation media is yet another advantage of agar. With respwect to the fact that ZnO-NPs may release toxic ions that can be more toxic than the nanoparticles themselves, the experiments with ZnO-NPs and soluble zinc chloride were performed in parallel.

Materials and methods

Chemicals

ZnO-NPs powder with a purity of 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; the particles being spherical with the hexagonal crystalline structure of zincite. Zinc chloride, $ZnCl_2$, purchased from PENTA (Czech Republic) was of *p.a.* grade and used in the tests evaluating the toxicity of zinc cations. Borax, $Na_4P_2O_7 \cdot 10H_2O$, with a purity of ≥ 99 % and obtained from Sigma-Aldrich served as a dispersant. Sodium humate originating from weathered brown coal and called oxyhumolite were obtained from Humatex (Czech Republic). Peat purchased from Agro CS (Czech Republic) was sifted through a sieve with a mesh size of 2 mm and then dried.

Test species

The culture of *Enchytraeus crypticus* was maintained in plastic Petri dishes with ventilation (Fisherbrand, PS, aseptic, 90 × 14.2 mm, Fisher Scientific, the 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 fed with finely ground oatmeal twice a week. Adults with a well-developed clitellum were used for the tests.

Preparation of exposure media

Experiments with $ZnCl_2$

The effect of zinc was tested in three different agar-based exposure media – pure agar, agar modified with sodium humate (SH, 0.1%), and agar modified with the addition of peat (1%). Exposure media was prepared by adding the dry agar (1.5%) and soil constituents to distilled water (negative control) or to $ZnCl_2$ solution in distilled water. The pH value of the mixtures was adjusted to 7 with the aid of $CaCO_3$ (Lach-Ner, Czech Republic). Mixtures were then vigorously stirred with a magnetic stirrer at 95 ± 5 °C for 30 min. The hot agarose medium was then poured into plastic Petri dishes with ventilation of both the bottom and the lid in order to prevent the escape of the test organism from the zinc containing environment. To facilitate faster penetration of organisms into the medium, notches were created in the cooled agar using a scalpel.

The tested concentrations of zinc in pure agar were: 0; 1; 10; 20; 30 and 60 mg kg^{-1} of agar (wet weight). This concentration range was chosen because an LC_{50} (statistically derived dose at which 50 % of the test organisms is expected to die) of 37.2 mg Zn kg^{-1} in pure agar was found in the previous study [17]. The concentration range tested in the presence of HA was broader (0; 1; 25; 50; 100; 250) than that of pure agar because it was expected that soil constituents would influence the bioavailability of zinc.

Experiments with ZnO-NPs

The preparation of the exposure media was different than in the case of the experiments with $ZnCl_2$. The spiking method, described in [17], was used with certain modifications. The respective procedure was based on the preparation of stabilized colloidal solution of nanoparticles in demineralized water in the presence of a dispersant. Solutions were added dropwise to hot agar 15 min. after reaching the temperature of 95 ± 5 °C. This temperature was maintained for additional 15 min. Subsequently, the procedure was the same as described in the previous sub-chapter; the dispersant being also added to a control group. The tested concentrations were 0; 50; 100; 250; 500; 1 000 mg ZnO-NP kg^{-1} of agar (0; 40; 80; 201; 402; 804 mg Zn kg^{-1} of agar).

Characterization of zinc oxide nanoparticles

After optimization of the key steps, the following procedure for the preparation of nanoparticles colloidal solutions was adopted: solutions for all the experiments (~ 50–1 000 mg kg^{-1}) were prepared using sonication for 45 min with addition of $Na_4P_2O_7 \cdot 10H_2O$ (0.02%) as a dispersant. The hydrodynamic diameter was measured using dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern, UK).

Samples of the agar media with ZnO-NPs concentration of 50 and 1 000 mg kg^{-1} prepared in all three modifications were characterized by scanning electron microscopy (SEM; model "JSM-5500LV", JEOL, Japan) using energy dispersive X-ray microanalyzer IXRF systems (with detector GRESHAM Sirius 10). The procedure for the sample preparation and analysis conditions are described in the previous study [17].

Design of ecotoxicity tests

Twenty adult worms, with visible eggs in the clitellum region, were placed into each Petri dish and maintained in the dark inside climate-controlled box 21 ± 2 °C for a period of 96 h. Three independent replicates were performed for each concentration level tested, including the negative control. The end-point of the tests was defined via the mortality in the medium with a particular concentration of zinc versus the mortality in the negative assay.

For obtaining of the dose-response curves and for calculation of LC_{50} values, the module of nonlinear regression as a part of GraphPad Prism 7 software was used.

Results and discussion

Characterization of ZnO nanoparticles

The hydrodynamic diameter in the nanoparticles colloidal solutions (~ 50–1 000 mg kg^{-1}) was measured using DLS. Considering the large differences in ZnO-NPs concentrations, the measurement showed a narrow range of the diameter. The range of nanoparticles effective diameter was 226–310 nm.

SEM with EDX microanalyzer was used for the characterization of nanoparticles in the agars. SEM images of agar with ZnO-NPs concentrations 50 (left column) and 1 000 mg kg^{-1} (right column) are shown in Fig. 1. In the samples of pure agar, the so-called "bubbles" of agar were observed (in Fig. 1, labeled as "1"). The analysis of the chemical composition revealed that these samples did not contain detectable zinc. There were detected preferable small agglomerates (size < 1 μm labeled as "2") for samples with 50 mg ZnO-NPs kg^{-1} and micrometric agglomerates (lateral size > 1 μm) for samples with 1 000 mg ZnO-NPs kg^{-1} (Fig. 1, labeled as "4"). Analysis of the sample area indicated that ZnO was dispersed throughout the entire volume of the agar in both samples (an example of a spot analyzed in an area is labeled as "3").

In the presence of 0.1% SH, larger agglomerates of ZnO (> 1 μm, 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 without agglomerates (labeled as "3") was higher than in the case of pure agar, indicating that a larger amount of zinc had been dispersed in the agar.

Especially in the agar with the addition of 1% peat it was possible to observe the peat structure. Zinc was very well dispersed throughout the volume of the area in both samples when very small amounts of ZnO-NPs agglomerates (< 1 μm) could be found in the sample with the ZnO-NPs concentration of 1 000 mg kg^{-1}. In the Fig. 1, SEM images (e,f) depict the peat formations or limestone particles used as a buffer.

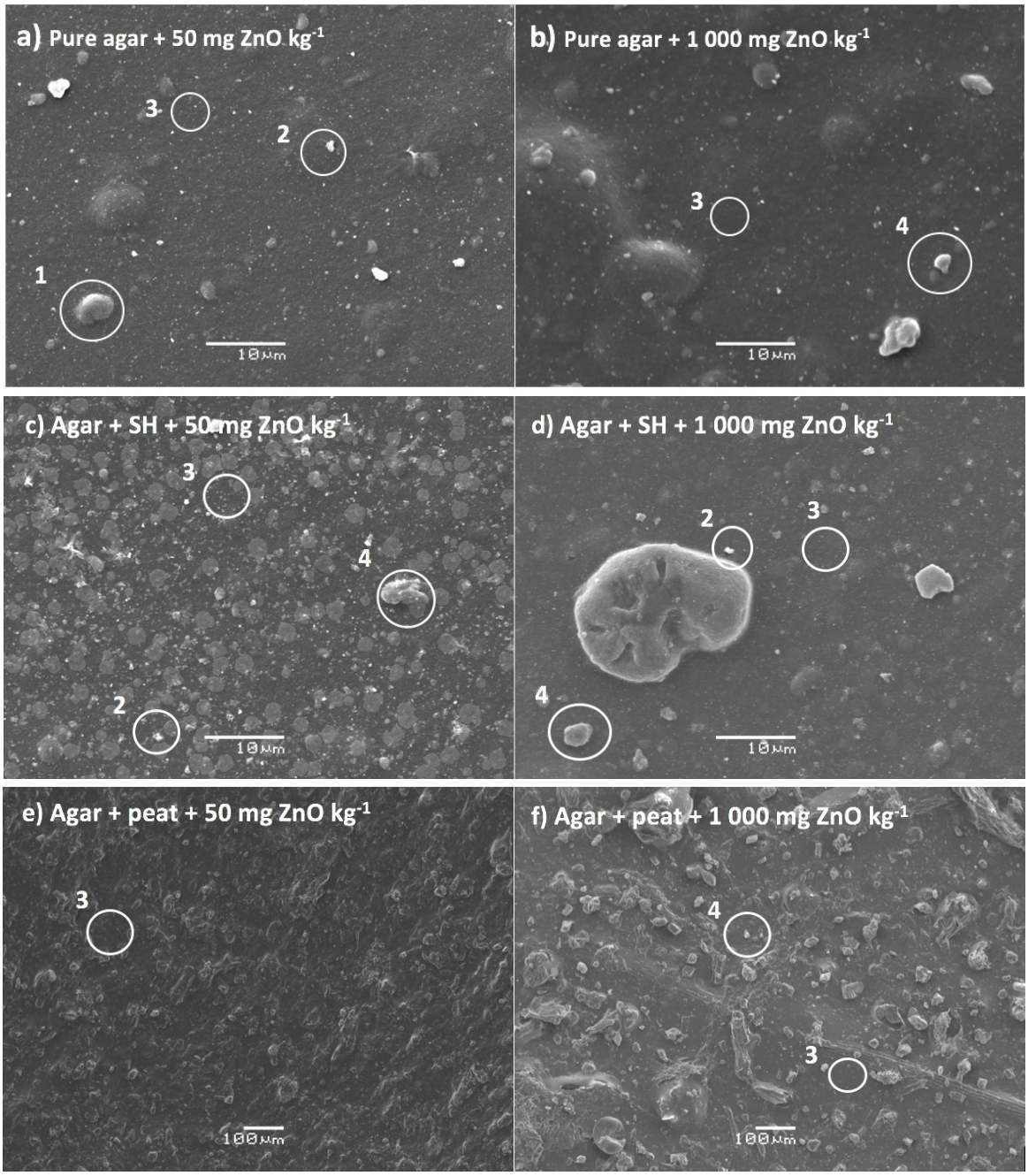

Fig. 1 *SEM images of agar with ZnO-NPs*

Examples of the objects observed and analyzed with EDX: (1) "bubble" of agar (2) small agglomerate (3) area (4) large agglomerate

Ecotoxicity tests

The dose-response curves describing the acute toxicity of zinc that originates from $ZnCl_2$ (a) and ZnO-NPs (b) are shown in Fig. 2. After 96 h exposure, the mortality in the exposure media increased with the increasing zinc concentration in all modifications of the exposure media. The calculated values for 96 h LC_{50} (corresponding to a 95% confidence interval) are shown in Tab. 1.

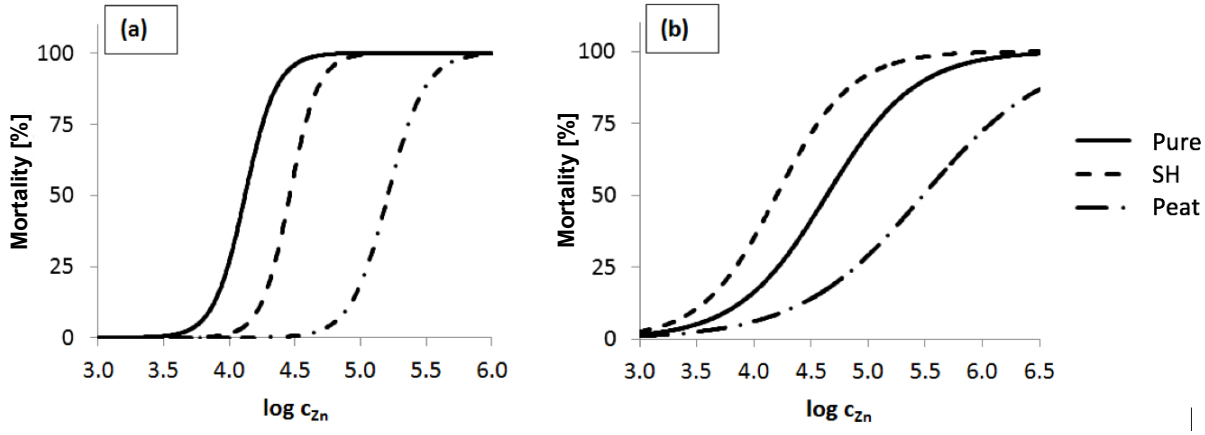

Fig. 2 *Dose-response curves describing the acute toxicity of zinc given by $ZnCl_2$ (a) and ZnO-NPs (b) in different exposure media*

Concentration of Zn expressed in 10^{-3} mg Zn kg^{-1} of agar

Table 1 *LC_{50} values for both form of zinc in three different exposure media*

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 SH	28.8 (27.7–30.0)	15.8 (3.3–36.9)
Agar with peat	161 (140–287)	304 (255–365)

Toxicity of zinc originating from $ZnCl_2$ was the highest in pure agar (13.2 mg kg^{-1}) followed by agar with SH (28.8 mg kg^{-1}), whereas the lowest toxicity was observed for agar with peat (161 mg kg^{-1}). Toxicity could be slightly decreased by adding SH but, in the presence of peat, the toxicity decreased by one order of magnitude. From such results, it is obvious that the addition of soluble SH did not reduce the bioavailability of zinc as much as the solid peat. Besides possible zinc complexation with humic acids, the solid portion of peat played an important role in influencing the fate of zinc in the agar. Also, structural and compositional differences of HA originating from different sources could lead to different features of HA [18]. For comparison, Koukal et al. [19] studied the influence of NOM presence on the toxicity of zinc to algae *Pseudokirchneriella subcapitata*. The three humic substances [soil and peat HA and Suwannee River Fulvic Acids (FA)] were used. Humic substances were added at two concentrations: 1 and 5 mg L^{-1}. It was found that HA markedly decreased the zinc bioavailability and toxicity to *P. subcapitata*, especially at 5 mg L^{-1}, whereas FA showed no significant effect at both concentrations. The authors have explained this difference so that the FA-metal complexes are far more labile, and dissociation may occur.

Metals dissociated from labile complexes are potentially more available, reactive and toxic. On the other hand, Kungolos et al. [20] reported that presence of HA did not significantly affect the toxicity of zinc to photobacterium *Vibrio fischeri* (Microtox test). The level of complexation of Zn(II) with HA was found to be low.

In case of ZnO-NPs, the toxicity was the highest in agar with SH (15.8 mg kg^{-1}) followed by pure agar (43.5 mg kg^{-1}) and the lowest toxicity was observed for agar with peat, too (namely: 304 mg kg^{-1}). As in the case of $ZnCl_2$, different composition of HA and the solid phase of peat had reduced toxicity of zinc almost by one order of magnitude compared to pure agar. Interestingly, there was even an increase in the toxicity of nanoparticles in the presence of SH compared to pure agar. SEM analysis showed that more zinc was dispersed in the volume of agar in the presence of SH. Apparently, HA stabilized ZnO-NPs which resulted in the increase of amount of well-dispersed nanoparticles. Also, ability of HA to increase dissolution of zinc (or other metals) was shown in other studies [21,22]. Similar behavior of ZnO-NPs in the presence of HA was also observed by Li et al. [16]. In their study, the biological response of ZnO-NPs on earthworms in the presence of HA was investigated using the filter paper contact method. It was found that the addition of HA had enhanced the dissolution of NPs. TEM images revealed smaller aggregates than in case of NPs without HA. The presence of HA, however, slightly decreased the toxicity of NPs. Akhil and Sudheer Khan [15] studied effect of HA on the toxicity of bare and capped ZnO nanoparticles (polyvinyl pyrrolidone, polyvinyl alcohol and ethylene glycol) on bacteria, algal, and crustacean systems. It was observed that the adsorption of HA decreased the hydrodynamic size and increased the stability for the bare NPs and NPs capped with polyvinyl pyrrolidone (PVP). As confirmed in our experiments, HA increased the toxicity of ZnO and ZnO-PVP NPs in all the systems. In contrast, Ouyang et al. [6] investigated the effects of HA on interactions between ZnO-NPs and *Pseudomonas putida* KT2440 biofilms at different maturity stages. Dissolved zinc ions significantly contributed to the overall toxicity and the presence of HA dramatically decreased the toxicity of NPs due to the binding of Zn(II) ions on HA.

Conclusions

A novel methodological approach has been used for testing the ecotoxicity of selected materials of the soil organism type. The use of agar instead of soil provides an advantage particularly in terms of simple and quick preparation of the experiment and easy preparation of the samples for the secondary characterization. The respective experimental setup allows one to study the nanoparticles in the real state in exposure media without further steps. Extraction of nanoparticles from the soil environment can often change the physicochemical

properties of the nanomaterial. In the agar medium, we easily demonstrated that the HA were able to influence bioavailability of zinc originating from both forms and that also the source of HA plays important role. The proposed experimental system is valuable in terms of the study of the soil components influencing nanomaterial features and toxicity. Though character of our agar-based exposure media is connected with the reduced degree of its environmental relevance, still, the lower complexity and the solid form of the system make agar a very beneficial type of exposure medium for studies of nanomaterials fate.

References

[1] Borm P., Klaessig F.C., Landry T.D., Moudgil B., Pauluhn J., Thomas K., Trottier R., Wood S.: Research strategies for safety evaluation of nanomaterials, part V: Role of dissolution in biological fate and effects of nanoscale particles. *Toxicological Sciences* **90** (2006) 23–32.

[2] Caballero-Guzman A., Nowack B.: A critical review of engineered nanomaterial release data: Are current data useful for material flow modeling? *Environmental Pollution* **213** (2016) 502–517.

[3] Quik J.T.K., Lynch I., van Hoecke K., Miermans C.J.H., De Schamphelaere K.A.C., Janssen C.R., Dawson K.A, Stuart M.A.C, van de Meent D.: Effect of natural organic matter on cerium dioxide nanoparticles settling in model fresh water. *Chemosphere* **81** (2010) 711–715.

[4] Liu X., Jin X., Cao B., Tang C.: Bactericidal activity of silver nanoparticles in environmentally relevant freshwater matrices: influences of organic matter and chelating agent. *Journal of Environmental Chemical Engineering* **2** (2014) 525–531.

[5] Wirth S.M., Lowry G.V., Tilton R.D.: Natural organic matter alters biofilm tolerance to silver nanoparticles and dissolved silver. *Environmental Science and Technology* **46** (2012) 12687–12696.

[6] Ouyang K., Yu X.Y., Zhu Y., Gao C., Huang Q., Cai P.: Effects of humic acid on the interactions between zinc oxide nanoparticles and bacterial biofilms. *Environmental Pollution* **231** (2017) 1104–1111.

[7] Stankus D.P., Lohse S.E., Hutchison J.E., Nason J.A.: Interactions between natural organic matter and gold nanoparticles stabilized with different organic capping agents. *Environmental Science and Technology* **45** (2011) 3238–3244.

[8] Yang K., Lin D.H., Xing B.S.: Interactions of humic acid with nanosized inorganic oxides. *Langmuir* **25** (2009) 3571–3576.

[9] Zhang Y., Chen Y.S., Westerhoff P., Crittenden J.: Impact of natural organic matter and divalent cations on the stability of aqueous nanoparticles. *Water Research* **17** (2009) 4249–4257.

[10] Edgington A.J., Roberts A.P., Taylor L.M., Alloy M.M., Reppert J., Rao A.M., Mao J.D., Klaine S.J.: The influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. *Environmental Toxicology and Chemistry* **29** (2010) 2511–2518.

[11] Fabrega J., Fawcett S.R, Renshaw J.C, Lead J.R.: Silver nanoparticle impact on bacterial growth: effect of pH, concentration, and organic matter. *Environmental Science and Technology* **43** (2009) 7285–7290.
[12] Ma H., Williams P.L., Diamond S.A.: Ecotoxicity of manufactured ZnO nanoparticles – A review. *Environmental Pollution* **172** (2013) 76–85.
[13] Gao J., Powers K., Wang Y., Zhou H., Roberts S.M., Moudgil B.M., Koopman B., Barber D.S.: Influence of Suwannee River humic acid on particle properties and toxicity of silver nanoparticles. *Chemosphere* **89** (2012) 96–101.
[14] Gunsolus I.L., Mousavi M.P.S., Hussein K., Bühlmann P., Haynes C.L.: Effects of humic and fulvic acids on silver nanoparticle stability, dissolution, and toxicity. *Environmental Science and Technology* **49** (2015) 8078–8086.
[15] Akhil K., Sudheer Khan S.: Effect of humic acid on the toxicity of bare and capped ZnO nanoparticles on bacteria, algal and crustacean systems. *Journal of Photochemistry and Photobiology B: Biology* **167** (2017) 136–149.
[16] Li L.Z., Zhou D.M., Peijnenburg W.J.G.M., van Gestel C.A.M., Jin S.Y., Wang Y.J., Wang P.: Toxicity of zinc oxide nanoparticles in the earthworm *Eisenia fetida* and subcellular fractionation of Zn. *Environment International* **37** (2011) 1098–1104.
[17] Hrdá K., Opršal J., Knotek P., Pouzar M., Vlček M.: Toxicity of zinc oxide nanoparticles to the annelid *Enchytraeus crypticus* in agar-based exposure media. *Chemical Papers* **70** (2016) 1512–1520.
[18] Malcolm R.L.: The uniqueness of humic substances in each of soil, stream and marine environments. *Analytica Chimica Acta* **232** (1990) 19–30.
[19] Koukal B., Gueguen C., Pardos M., Dominik J.: Influence of humic substances on the toxic effects of cadmium and zinc to the green alga *Pseudokirchneriella subcapitata*. *Chemosphere* **53** (2003) 953–961.
[20] Kungolos A., Samaras P., Tsiridis V., Petala M., Sakellaropoulos G.: Bioavailability and toxicity of heavy metals in the presence of natural organic matter. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering* **41** (2006) 1509–1517.
[21] Shao-Wei B., Mudunkotuwa I.A., Rupasinghe T., Grassian V.H.: Aggregation and dissolution of 4 nm ZnO nanoparticles in aqueous environments: influence of pH, ionic strength, size, and adsorption of humic acid. *Langmuir* **27** (2011) 6059–6068.
[22] Sanchez-Marin P., Lorenzo J.I., Blust R., Beiras R.: Humic acids increase dissolved lead bioavailability for marine invertebrates. *Environmental Science and Technology* **41** (2007) 5679–5684.