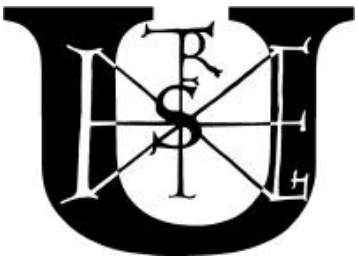

SZENT ISTVÁN UNIVERSITY

PHD THESIS

The ecotoxicological effect of nano and bulk ZnO on soil organisms

Kiss Lola Virág

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The doctoral school's

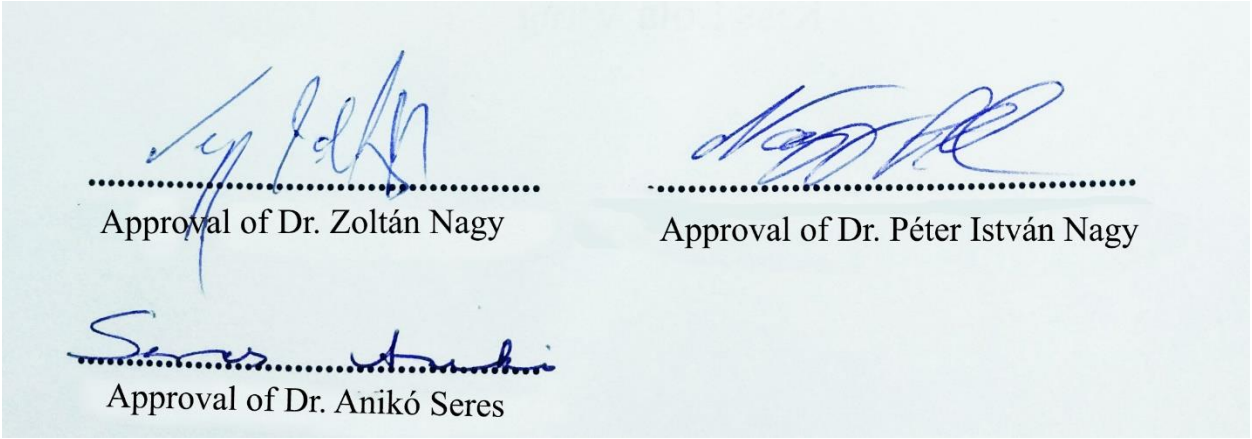
Name: SZIE PhD School of Biological Sciences

Discipline: Biological Sciences

Leader: Dr. Zoltán Nagy
professor, DSc Institute of Botany and Ecophysiology

Supervisor: Dr. Péter István Nagy
associate professor, PhD Institute of Zoological Sciences, Department of Zoology and Animal Ecology

Supervisor: Dr. Anikó Seres
assistant professor, PhD Institute of Zoological Sciences, Department of Zoology and Animal Ecology



1. INTRODUCTION AND OBJECTIVES

The widespread character of nanotechnology in every aspect of human life results in massive release of engineered nanomaterials to the environment. Nanomaterials have new physical and chemical properties as a result of their alteration in size. The first step for assessing the proper risk to the environment is understanding the link between these new physicochemical properties and their biological effects. Fortunately, the number of publications dealing with their side-effects increased exponentially, moreover, the modification of standard toxicity tests to fit for nanomaterials have been also started. However, the research results are difficult to compare and replicate due to the lack of standard methods. In addition, many testing errors can be found when examining available research. Incomplete characterization of the materials, the usage of inadequate concentrations or even omitting of necessary control groups can be typical.

For metal-based nanomaterials like zinc oxide (ZnO) is significantly important as their wide-range of commercial applications. In addition, their toxicity can result from multiple properties including photo-induced and normal dissolution of zinc ions, generation of reactive oxygen species (ROS) and other potential particle-specific effects like direct contact between the particles and the cells of an organism. Moreover, size relevant effects of ZnO nanoparticles (NPs) and excess release of Zn^{2+} can also result in the generation of ROS. Even so most studies suggest the ZnO NPs dissolution to ionic Zn playing the greatest role in eliciting toxicity. ZnO NPs are also among the most commonly used metal-oxides as applications include soil remediators, water cleaners, sunscreens, cosmetics, food additives and also potential cancer medicine. The release of ZnO NPs due to its varied applicability (for example water purification, soil remediation, medical use) is not expected to show a downward trend, despite the vast amount of data available on potential hazards and risks. This has made it an important task to reduce the toxic effects of ZnO NPs. A suitable method for this is to mitigate or even eliminate the negative effects in such a way that the beneficial properties are maintained with the help of various mitigating agents. The N-acetylcysteine (NAC) antioxidant could be suitable for this task. Moreover, the wide use of diverse engineered nanomaterials can also lead to release of different mixtures of nanomaterials into the environment. The possible interaction between these may have altered impacts on organisms as compared to the effects caused by individual NPs alone.

After being released to the soil environment, ZnO NP can be sorbed onto soil particles, react to organic materials and even be transported to groundwater. As a result of the large released amounts and a potential leaking into pore water, ZnO can pose a great risk to soil microfauna including nematodes. Therefore, it is very important to investigate the effects on soil organisms exposed to directly to ZnO NPs, preferably in the most environmentally relevant test medium. This way soil can be taken into account as an influencing factor.

An extensive amount of research material already exists concerned with the measuring of the toxic effects of ZnO NPs on nematodes, ZnO NPs were found to be toxic in the applied concentrations to nematodes in all cases.

In addition to the microfauna, members of a mesofauna are also heavily exposed to various types of soil contamination. Springtails (Collembola) have been used for ecotoxicology testing going back decades and hence this group has been involved in several studies with ZnO NPs. In these treatments, the survival of Collembola was found to remain unaffected by ZnO NPs at concentrations up to 6400 mg/kg Zn; however, the ZnO NPs did have an effect on reproduction, which was concentration dependent.

1.1. Objectives

- study of the effects of different test media in ecotoxicological test systems:

  - development of a more environmentally relevant test medium involving the nematode *Panagrellus redivivus* in the presence of 15 nm and 140 nm ZnO;
  - investigation on the effects of different routes of exposure (via cuticle and oral and oral only) on the toxicity of the two ZnO particles with the springtail *Folsomia candida*;

- detection of particle size dependent toxicity, understanding and investigating mechanisms of action of used ZnO particles:

  - using N-acetylcystein mitigation agent to highlight differences in toxicity mechanism;
  - carrying out interaction experiments to simulate changes in toxicity due to environmental mixing and to specifically investigate particle size toxic effects;
  - developing a practical method for measuring the amount of produced reactive oxygen species in *P. redivivus* cells;
  - investigation on the toxicity mechanism of particle size by the measurement of the produced reactive oxygen species.

2. MATERIALS AND METHODES

2.1. Examination of test compounds

Two ZnO particles of different nominal particle sizes were applied as (i) 10–30 (referred to as 15 nm average particle size) nm with purity 99 + % (spherical) and (ii) 80–200 (referred to as 140—average particle size) nm ZnO with purity 99.9 + % (irregular). Both materials were purchased from US Research Nanomaterials, Inc. The compounds were produced by the wet chemical method (a group of methods used for producing nano-dispersed inorganic powders from aqueous and non-aqueous solutions) and there was no surface treatment on them. The specific surface areas (SSA) were 20–60 m^2/g and 4.8–6.8 m^2/g for 15 nm and 140 nm ZnO, respectively. The particle shape of 15 nm ZnO was nearly spherical and that of the 140 nm ZnO was irregular. $ZnCl_2$ was used as a free ionic positive control. For the mitigation experiments, N-acetylcysteine was the mitigation agent. Both were purchased from Sigma-Aldrich.

The different particle-sized ZnO was characterized by a variety of analytical techniques. The primary particle size and particle morphology were measured by scanning electron microscopy (SEM, FEI Quanta 3D, ELTE, Hungary). For each of the materials and for the mixture of both, images were recorded and the average size and the size distribution were ascertained by measuring approximately 100 particles from representative images by the Image J software package. The ZnO particles were also checked in soil solution after 48 h and with the addition of N-acetylcysteine after 24 h incubation by SEM.

The dissolution of the materials in suspensions (Milli-Q water and soil solution) at two time points (24, 48 h) was assessed by centrifugation, followed by chemical analysis of complex Zn^{2+} using inductively coupled plasma atomic emission spectroscopy (ICP-OES, Horiba Jobin–Yvon Activa-M, SZIE, Hungary).

2.2. Studing of the effects of various test media in the presence of differently sized ZnO particles

2.2.1. *Panagrellus redivivus* toxicity assay: various test media

Panagrellus redivivus was selected to represent microfauna in this experiment. Stock culture is held in a thermostat chamber in Department of Zoology and Animal Ecology, Szent István University. In the present experiment, 5–5 adult females were used in an acute mortality test in two different test media, Milli-Q water and soil solution. Apart from the exposure time (Milli-Q water—24 h; soil solution—24, 48 h), both tests were performed in the same way. No standard guideline is available for mortality testing of this species; therefore, a maximum mortality of 20% in the control group was chosen as the validity criteria. The applied concentrations were 0.63, 1.26, 2.51, 5.02 and 10.04 mg/l Zn in the case of Milli-Q water. Higher concentrations were also used in the soil solution (514.20, 1028.40, 2056.80, 4113.61, 8227.22 and 16454.5 mg/l Zn) based on pre-test results, because no significant effect was found at the same concentrations except in the case of $ZnCl_2$. Four replicates for each concentration and control were applied. The experiment was performed in 96-well microplates (Bioster S.p.A., Italy).

A group of animals was randomly sampled from the stock culture into a counter filled with Milli-Q water. From here, I selected the female specimens by pipette. Since 90% of the stock animals are female, care should be taken to ensure that no male animal is included in the experimental system. The female genitalia is located in the midline of the abdomen, while the males' is located in the back of the body, where the short, curved spicule is clearly visible. In addition, males are generally somewhat smaller than females. Prior to animal placement, 100 µl of Milli-Q water or soil solution was pipetted onto microplates to create a humid environment. Then, in both experiments, animals were relocated with 2x30 µl Milli-Q water in each well. Since the wells contained some liquid by the time the solutions were added, I prepared twice the nominal concentrations before the experiments. After that, 160 µl of the test solution or Milli-Q water, in the case of the control group, were added to the test system to reach the final amount of 320 µl

liquid. The microplates were incubated in a thermostat under dark conditions at 20 ± 1 Cº. Surviving specimens were counted at the end of the experiment under a transmission stereomicroscope (Olympus SZH 10).

2.2.1.1. Method development

According to the literature, animals were placed on 96-well microtiter plates with a glass pipette. Since I found this method too time consuming and inaccurate, I switched to using a 5-200 µl pipette. This way, I was able to place the animals in the experimental system with the correct amount of liquid, and after a little practice, the transfer itself becomes easier, which was very important when setting up large numbers of experiments.

2.2.2. *Folsomia candida* toxicity assay: various test media and way of exposure

Folsomia candida is often used as a standard test soil organism in soil toxicological experiments. Specimens from the laboratory stock culture of Szent István University Department of Zoology and Animal Ecology were examined in two different test media, plaster of paris and artificial soil. In the case of plaster of paris, the OECD 232 guideline was modified based on literature. The same mixture was used in the experiment as in the case of the stock cultures (200 g plaster of paris/ 200 g water/ 10 g activated carbon). Although the animals can partially consume the activated carbon from the plaster of paris test medium, this mixture found to be suitable based on preliminary experiments. The survival of cultures without the addition of activated carbon is reduced, and the lack of carbon is also make the test system very difficult to read at the end of the experiment. Compounds were introduced into the food instead of the test medium, to reach exclusively per os exposition. The food was mixed with a working solution (to 0.5 g yeast 1.5 ml solution) and after that placed on a filter paper. Fresh food was added once a week. The artificial soil was established based on the OECD 232 guideline. 25 g amount of soil was placed into the vessels, and then 5 ml of compound or distilled water (in the control) was added. The same concentrations were used for the springtail tests, namely: 160.69, 321.37, 624.75, 1285.5 and 2571 mg/kg Zn. These concentrations are equal to 803.44, 1606.87, 3213.75, 6427.5 and 12855 mg/l Zn when recalculated for the quantity of the applied solution in soil and 3213.75, 6427.5, 12855, 25710 and 51420 mg/l Zn in plaster of paris. Four replicates for each concentration and the control were prepared. Subsequently, ten specimens (10–12 days old) were added in both cases to the test vessels. At the end of the experiments, the number of the surviving adult animals and the juveniles was counted. The exposure time was 28 days for both tests.

2.3. Understanding and investigating mechanisms of action

2.3.1. The mitigation effect of N-acetylcysteine on *Panagrellus redivivus*

Toxicity mitigation assays were carried out based on the modifications of previously published methods. 5-5 adult females were used in acute mortality tests in Milli-Q water test media. The tests were performed with both ZnO particle and $ZnCl_2$ pure and in the presence of NAC. The applied concentrations were 0.32, 0.63, 1.26, 2.51, 5.02 and 10.04 mg Zn/l in most the case, except for the ionic zinc form. For $ZnCl_2$ the concentrations were set up based on the measured mean dissolution rate (0.13, 0.26, 0.53, 1.06, 2.12 and 4.23 mg Zn/l). These concentrations correlated with the concentrations used in the ZnO toxicity tests. Four replicates for each concentration and control were applied. Furthermore, a negative control (320 µl Milli-Q water) and a control containing NAC (160 µl Milli-Q water and 160 µl NAC) were also set up. The experiment was performed in 96-well microplates (Bioster S.p.A., Italy). A 10 mg / l stock solution was prepared, from that 100 µl pipetted into each cuvette. In the mitigation experiments, the animals extracted from the stock culture were not placed in Milli-Q water but in 10 mg/l NAC solution and were selected with 2x30 µl or once with 60 µl liquid. After all the animals have been as described in *2.2.1. Panagrellus redivivus toxicity assay* chapter, 160 µl of working solution was added to each well to achieve a final concentration of 5 mg / L NAC. The microplates were

incubated in a thermostat under dark conditions at 20 ± 1 ºC. Surviving specimens were counted after 24 hours under a transmission stereomicroscope (Olympus SZH 10). N-acetylcysteine (NAC) was used to elucidate the relevance of intracellular ROS generation as well as dissolved zinc ions in ZnO toxicity. NAC was mean to reduce the toxicity of ZnO nanoparticles in all cases.

2.3.2. Interaction between ZnO nanoparticles

P. redivivus acute mortality test was used in the experiments investigating the interaction between ZnO nanoparticles. The concentration series were made from the 50-50% mixture of 15 nm and 140 nm ZnO. Experiments were carried out the same way as describe in *2.2.1. Panagrellus redivivus toxicity assay* and *2.3.1. The mitigation effect of N-acetylcysteine on Panagrellus redivivus* chapters. In the preliminary experiments, only the three highest concentrations were tested (2.51; 5.02 and 10.04 mg / l Zn) on their own and also in addition with NAC to see if there was any valid result. Since there was a strong difference between the toxicity of the mixture and of the original substances, mainly with the addition of NAC, the entire experiment was repeated using the whole concentration series (0.32, 0.63; 1.26; 2.51; 5.02 and 10.04 mg / L Zn). After 24 hours of incubation, the microplate was examined under a stereomicroscope. Milli-Q water was used as a negative control.

2.3.3. Measuring the intracellular reactive oxygen species generation

Intracellular ROS production was measured using an aminophenyl fluorescein (APF; Thermo-Fischer) assay, with the help of Dr. András Ács from the Department of Environmental Security, Szent István University. APF is a relatively new and more specific indicator for ROS measurement than hitherto used dyes (such as 2 ', 7'-dichlorodihydrofluorescein diacetate). It is more tolerant of light-induced oxidation and becomes fluorescent in the presence of hydroxyl radical (OH ·), preoxynitrite anion (ONOO-) and hypochlorite anion (OCl-). APF reacts three times more strongly to hydroxyl radicals than to other ROS radicals, eg superoxide ($\cdot O_2$-), hydrogen peroxide (H_2O_2) or singlet oxygen (O^1_2). Many studies have suggested that the photocatalytic and antibacterial properties of nanoparticle oxides are mainly due to free and surface-bound hydroxyl radicals, although superoxide and hydrogen peroxide play an important role in the processes. Thus, we can assume that OH· production is a representative of total ROS formation by ZnO NPs.

No standard guideline is available for measuring intracellular ROS with APF, therefore a modified method was used based on other ROS measuring methods. The measurement were set up similarly to the toxicity assay except the exposure time was only 3 hour and for all six types of compounds, three concentrations were tested: 2.51, 5.02 and 10.04 mg Zn/l (in Milli-Q water). Instead of 96 well microplate the test compounds were put inside Eppendorf tubes to ensure the proper recovering of the nematode samples. After the incubation time, all the replicates from one treatment were put into one tube (40 animal/tube). Worms were suspended in 1.5 ml phosphate-buffered saline buffer (PBS, pH 7.2). The tubes were centrifuged at 600g for 5 min, then the PBS was aspirated out. Only 80 µl liquid was left inside the tubes with the animals. After that, from the previously prepared APF stock (10 µM in PBS) 160 µl has been immediately added to the samples. Worms were broken up with a homogenizer for 2 min (Qiagen Tissuelyser LT homo). Span the mixture down for 12 min at 17 000g and separate the worm lysate from the carcass pellet (Qhaus Fe5718R). The protein concentration was determined from 15 µl worm lysate (30 µl from the samples + APF stock) using the Bradford Assey following manufacturer's instructions.

Two replicates were used per treatment. The exposure was conducted in 96-well black microplate for APF test. 75 µl sample + APF was supplemented with 25 µl PBS to reach 100 µl per well. As for the blank (50 µl PBS) and the positive control (50 µl H_2O_2, 50 mM) 50-50 µl APF was added to every replicate. This method needed 30 min incubation before the reading (dark condition, 37 ºC). The samples were read at 490 nm/515 nm on microplate reader (Thermo Scientific Varioskan Lux).

2.3.3.1. Method development

The method suggested by the protocols from literature proved to be unsuitable. According to the literature, only 5 to 20 adult nematodes are needed to the preparation of the lysate. Based on my experience, this amount was not enough for either fluorescence measurement or protein measurement, so the number was increased to 40. In addition, the protocol recommends removing supernatant above the animal 100 µl of liquid remaining in the tubes. This is not feasible since the amount of liquid remaining in the Eppendorf tube cannot be accurately determined. Even though animals settle to the bottom of the tube, for such a small amount it is inevitable that they enter the pipette. Another study, on the other hand, suggests a more feasible method, by pipetting the animals onto a glass slide, but their recommended a volume of 10 µl which is very low for the 40 animals used in my experiments. So, from my own experience, the animals can be moved using 80 µl of liquid. Both protocols and another study suggest adding an indicator to the sample after lysis, but in our tests it was found that too much time elapses between incubation with the test material and measurement, so that animals still alive are able to degrade the produced reactive oxygen species. Therefore, the APF was added immediately after the test end and lysed with it, thus preventing the breakdown of the ROS. After the developments, the method became usable and the protein and fluorescence measurements of the samples were successful. The developed method was repeated 6 times to make sure it was functional.

3. RESULTS

3.1. Studing of the effects of various test media in the presence of differently sized ZnO particles

3.1.1. Particle characterization by SEM

From approximately 100 particles, an average primary particle size of 59 ± 31 and 174 ± 138 (Mean ± SD, n = 100) nm was calculated, in contrast to the nominal average particle size of 15 nm ZnO and 140 nm ZnO, respectively. Particle distributions have shown that both particle sized ZnO contained nano and bulk particles. However, when calculated from the column width, it can be said that the first material had more than 50% nano- and the second material had more than 50% bulk particles in them. The most common particle size values were in the 34-51 nm and 37-97 nm range for the 15 nm and 140 nm ZnO, respectively.

Further recordings revealed that ZnO NPs bound to the organic components in the soil solution and made larger aggregates with the soil particles.

3.1.2. Zinc ion dissolution

The complex Zn^{2+} dissolution of 15 nm and 140 nm ZnO in Milli-Q water and soil solution was measured. There was significantly less amount of dissolved Zn^{2+} in the soil solution than in the Milli-Q water, both for 15 nm and 140 nm ZnO. After 24 h the dissolution halved for both compound, after 48 h 25% and ~60% was the rate of the decline for 15 nm and 140 nm ZnO, respectively. In Milli-Q water, there was no difference between the dissolution for both compounds after 24 h. However, after 48 h for the 15 nm ZnO, the dissolution increased 1.5 times then for the 140 nm ZnO. In the soil solution, the dissolved amount was significantly greater for 15 nm ZnO than for 140 nm ZnO after 24 h (about 35%). In contrast, after 48 h, the trend reversed and found higher levels of dissolved zinc in the case of the bulk material. One and a half times of the 15 nm ZnO was dissolved from the bulk material. In the case of 140 nm ZnO, the increasing intensity was similar to that observed in the Milli-Q water after 48 h. However, in the case of 15 nm ZnO, the dissolved Zn^{2+} significantly decreased after 48 h.

The pH values of 15 nm ZnO, 140 nm ZnO and $ZnCl_2$ working solutions were between 6 and 6.5.

3.1.3. *Panagrellus redivivus* toxicity assay: various test media

In the Milli-Q water test medium, all of the tested compounds were significantly more toxic than in the soil solution. The LOEC (lowest observed effect concentration) values were 0.32 mg/l Zn and 1.26 mg/l Zn for both ZnO and $ZnCl_2$, respectively. In contrast, when using the soil solution, the ZnO compounds had no effect in the same concentrations, but only in the higher ones after 48 h. Thus, 1028.4 mg/l Zn were the LOEC value for the 15 nm ZnO and 140 nm. The $ZnCl_2$ proved to be more lethal, as in the higher concentration series, all of the nematodes were killed (LOEC: 10.04 mg/l Zn). In the soil solution, the toxicity of the two ZnO forms was different, as shown by LC_{50} values as well (15 nm: 3645.3 mg/l Zn; 140 nm: 785.1 mg/l Zn). The 140 nm ZnO was more toxic. In Milli-Q water there was no difference in toxicity between the two compounds.

3.1.4. *Folsomia candida* toxicity assay: various test media and way of exposure

3.1.4.1. Mortality tests

The exposure routes and test media (on plaster of paris and in artificial soil media) in the *F. candida* toxicity test significantly influenced the mortality of the animals. When using 15 nm ZnO and $ZnCl_2$, mortality was significantly higher in artificial soil (compound mixed in the soil)

than on plaster of paris (compound mixed in the food). In the mortality test, no difference in particle size toxicity was observed in either medium. On the other hand, $ZnCl_2$ produced significantly higher mortality in artificial soil than the two ZnO compound. At the highest concentration (2571 mg/kg Zn), all animals were killed in the $ZnCl_2$ treatment. This result was supported by the LC_{50} values (15 nm: 8228.2 mg/kg Zn, 140 nm: 8170 mg/kg Zn, $ZnCl_2$: 557.31 mg/kg Zn).

3.1.4.2. Reproductivity tests

In the case of reproduction, the effect of the exposure routes showed a similar tendency, in this case in all the materials had stronger toxicity in artificial soil than on the plaster of paris medium. When mixed in the food on plaster of paris, the 15 nm and 140 nm ZnO particles had a mild and $ZnCl_2$ had a strong reproduction inhibitory effect (LOAEC: 15 nm and 140 nm ZnO - 2571 mg/kg Zn; $ZnCl_2$ - 321.28 mg/kg Zn). Furthermore, there was a significant difference between the toxicity of 140 nm and $ZnCl_2$. In artificial soil, reproduction decreased in a concentration-dependent manner. For the first time in the *F. candida* assay, particle size-dependent toxicity was observed, with 140 nm ZnO having a stronger inhibitory effect on reproduction. When mixed in the soil, both ZnO particles had a less toxic effect than $ZnCl_2$. Thus, $ZnCl_2$ inhibited reproductive processes most of all in artificial soil media during the reproduction test. These effects are also reflected in the EC_{50} values (15 nm: 1543.9 mg/l Zn, 140 nm: 393.2 mg/l Zn, $ZnCl_2$: 157.9 mg/l Zn).

3.2. Understanding and investigating mechanisms of action

3.2.1. Particle characterization by SEM

The binary mixture of 15nm and 140nm ZnO individually and all three materials (15 nm, 140 nm, binary mixture) with N-acetylcysteine were investigated. The average size measured by measuring the diameter of 100 particles was 130 ± 118 nm (mean ± SD, n = 100). It can be seen that the most common particle size by mode is 30 nm, but also larger aggregates (877 nm) may be present. In addition, the mixture clearly shows that both forms (spherical, irregular) are present in the samples. The addition of NAC did not significantly change the size or morphology of the materials. Apparently there was no coating around the materials by the NAC.

3.2.2. Zinc ion dissolution

There was no significant difference in the complex Zn^{2+} dissolution associated with mitigation studies between the two pure ZnO particles of different size. On the other hand, significantly more zinc ions (~30-40%) were dissolved from the binary mixture of the two substances. An increase in zinc dissolution was also observed with the addition of NAC to all test materials. There was a significant difference for 15 nm and 140 nm ZnO (20-30%), and a less strong but also significant difference for the mixture (4%) compared to the dissolution values of the untreated pairs of materials. Furthermore, in all cases significant difference was found between the NAC-treated compounds. For the 15 nm and the mixture, this difference was relatively slight (~ 3%).

3.2.3. The mitigation effect of N-acetylcysteine on *Panagrellus redivivus*

When testing the pure materials, similarly to the test media assay, no particle size-dependent toxicity was observed. $ZnCl_2$ was significantly less toxic than the two ZnO particles in the concentration series based on the dissolved zinc content. The addition of NAC significantly reduced the toxic effects of 15 nm ZnO. In the case of 140 nm ZnO a slight mitigating effect was observed. The mitigating effect showed a decreasing tendency above 2.51 mg/l and 1.26 mg/l for

15 nm and 140 nm ZnO, respectively. In contrast, when using $ZnCl_2$, mitigation was only observed if the highest concentration (4.23 mg / l Zn) was excluded. The two different particle sizes were also affected differently by the mitigation treatment. Notwithstanding the two highest concentrations, where none of the substances had a mitigating effect, a greater decrease in toxicity was observed in the presence of 15 nm ZnO than at 140 nm ZnO. These results are also apparent from the LC_{50} values (Pure: 15 nm: 1.85 mg/l Zn; 2.66 mg/l Zn; Addition of NAC: 15 nm: 14.091 mg/l Zn; 140 nm: 10.73 mg/l Zn).

3.2.4. Interaction between ZnO nanoparticles

A synergistic increase in toxicity was observed when using the binary mixture of the two particle sized ZnO. Compared to the 15 nm ZnO particle, the mixture proved to be significantly more toxic, even when used without the addition of NAC. Moreover, this difference in toxicity was further increased by the addition of NAC, thus the toxic effect of the mixture was not only stronger than that of the 15 nm but also than that of the 140 nm ZnO particles alone. There was no statistically demonstrable mitigating effect of the antioxidant on the mixture when studying all of the concentrations, although up to 1.26 mg/l Zn a slight decrease in toxicity was observed from the mixture without NAC. This can also be supported by LOAEC values (Pure: 0.31 mg/l; NAC: 1.26 mg/l). The mitigating effect was less for the mixture than for the two ZnO particles alone, as can be seen from the LC_{50} values (Pure: 0.65 mg/l Zn; Addition of NAC: 1.62 mg/l Zn).

3.2.5. Measuring the intracellular reactive oxygen species generation

The most common ROS content was 0.59-0.63 μM/mg (peak value: 1.07 μM/mg) in the Milli-Q water control groups and 0.74 -1.09 μM/mg in the NAC control groups. For the sake of comparability of the data series, I averaged each one. Thus, when performing statistical analyzes, I have already worked with the corrected values based on the means (Milli Q aqueous control: 0.69 μM/mg; NAC control: 0.97 μM/mg). Among the three measured pure materials, the highest ROS production was observed for the mixture, and the lowest for the 140 nm ZnO. Therefore, 140 nm ZnO was significantly different from the mixture and 15 nm ZnO. A concentration-dependent ROS increase was observed in the case of 15 nm ZnO and the binary mixture. The addition of NAC in the control groups resulted in higher oxygen radical production. In the presence of NAC, a completely different tendency was observed than that of the materials used alone. There was no significant difference between the materials. Both for 140 nm ZnO and for the mixture, a sharp increase in ROS production was observed at 2.51 mg/l Zn concentration, followed by a strong decrease.

4. NEW SCIENTIFIC RESULTS

- I have shown that ZnO particles of different particle sizes (15 nm and 140 nm) are less toxic to the *Panagrellus redivivus* nematode test species in soil solution than in Milli-Q water. This is due to the relationship between the ZnO particles and the organic and inorganic components of the soil. In addition, I demonstrated that oral exposure on plaster of paris media to the *Folsomia candida* collembola test species decreases the toxicity of 15 nm ZnO for mortality, 15 and 140 nm ZnO for reproduction compared to artificial soil. Collembolas in soil are more susceptible to cuticular exposure and are likely to avoid contaminated food on the plaster of paris.

- In mitigation studies, I found that N-acetylcysteine has a toxicity mitigation effect on the *Panagrellus redivivus* test species in the presence of 15 nm and 140 nm ZnO. However, I have also shown that N-acetylcysteine has a very low mitigating effect on $ZnCl_2$ and the binary mixture of the two nanomaterials (15 nm and 140 nm ZnO) for the same test species.

- Dissolved Zn assay demonstrated that in addition to the toxic effects caused by Zn^{2+}, there are additional particle size specific effects of the tested substances (15 nm and 140 nm ZnO). This effect may be due to the formation of ROS independent of ionic toxicity and the direct physical interaction of the particles.

- I proved that in several cases 140 nm ZnO was more toxic than the smaller particle sized ZnO (soil solution - *P. redidivus*; artificial soil - *F. candida*; mitigation studies - *P. redivivus*). This may be due to the faster and higher rated binding of 15nm ZnO particles to the soil particles, morphological differences and to the fact that N-acetylcysteine was less able to make complexes with 140 nm ZnO than with smaller particle sized one.

- I have shown that the binary mixture of the two substances (15 nm and 140 nm ZnO) is synergistically more toxic than each particle size groups applied alone.

- With my additions, a method has been developed for measuring intracellular reactive oxygen species (ROS) production in the *Panagrellus redivivus* test species. By this method, I have shown that ZnO particles cause reactive oxygen species production in the nematode *Panagrellus redivivus* in all cases, and this amount was highest for the binary mixture and lowest for 140 nm ZnO. The ROS inducing effect of ZnO NPs in nematode test organism has not been investigated in the literature.

5. DISSCUSSION

5.1. Studying the effects of various test media in the presence of differently sized ZnO particles

In this series of tests, the effects of two different test media were examined on an indicator species of the micro- and mesofauna. Significant difference was found between the two test media in the case of both species. The toxicity of the tested ZnO substances was reduced with the use of plaster of paris medium (*Folsomia candida*) and soil solution (*Panagrellus redidivus*). Based on these results, it can be demonstrated that the test medium significantly influences the outcome of the toxicity tests.

5.1.1. *Panagrellus redivivus* toxicity assay

Although several studies have investigated the toxicity of ZnO NPs on various environmentally relevant soil organisms and considered the factors that can affect the toxicity like aggregation, surface coating, pH, dissolution, mode of exposure, morphology and photo-induced effects, little effort has been made to test the same compound in different test media.

In the current experiments, at the used concentrations, all test substances proved to be toxic to *Panagrellus redivivus*, despite the fact that this species was the least sensitive species in comparison with other two bacterial nematodes in the literature. The literature lack the acute toxicity data for this species, however for other bacterivore nematode (*C. elegans*) the 24 hour LC_{50} values are between 0.32-111 mg/l Zn in aqueous media. The results of the present study showed that the LC_{50} values in Milli-Q water were comparable to the lower values. Soil solution was used as a test medium in quite a few studies. According to literature data, zinc uptake in the presence of soil solution is lower as compared to water. In the present study, ZnO particles were less toxic in the soil solution. This could be due to the interaction of the dissolved zinc content and the particles with some components of the soil solution, such as fulvic acid, humic acid and tannic acid. Of the three, tannic acid is considered the most successful substance in decreasing the ZnO NPs toxicity. In the present experiment, both ZnO particles had less dissolved zinc content in the soil solution. Dissolution may have been influenced by the pH of the soil solution (Milli-Q in water: pH 6-6.5, Soil solution: pH 7-7.5), since the lowest amount of dissolved ions are presented on neutral pH. The more intense aggregation could also be the reason for this phenomenon, as proved by the $ZnCl_2$ tests (100% mortality in the higher concentration series when the ZnO particles had less effect on the nematodes) and the SEM images where we could see the ZnO particles binding to the soil particles. Based on my experiments, the soil solution reduces the toxic effects of the tested 15 nm and 140 nm ZnO on *Panagrellus redivivus* species to a medium strong extent.

5.1.2. *Folsomia candida* toxicity assay

According to literature data, the ZnO NPs have a low or no effect on mortality of the springtails up to 6400 mg/l Zn concentrations. So far, scientists mainly attribute the toxic effects to the dissolved ions, finding that the dissolved zinc forms have a stronger effect than the nanoparticles at the same concentrations. In addition, the pH of the medium and the organic matter content have a strong influence on the solubility of zinc ions and thus toxicity. These findings are confirmed by an avoidance test in the literature where Collembola avoided the $ZnCl_2$-contaminated soil more than the ZnO NPs. These observations are also supported by the present study, although it appears from further experiments that the toxicity of ZnO NPs is not only explained by the dissolution of Zn^{2+}. Other published experiments used the same method to test different exposure routes as in the present study, but they applied different compounds (Cd, Cu, Pb, Zn). They found

that the tested metals were more toxic when incorporated into the soil in standard tests rather than added to the food of *F. candida*. In the soil, springtails face with more cuticular exposure more typically. Moreover, it is suggested that they may avoid the contaminated food in the experiment with plaster of paris. The 15 nm - for mortality - 15 and 140 nm ZnO - for reproduction - were significantly less toxic on the plaster of paris than in artificial soil in the present study as well.

5.1.3. The mitigation effect of N-acetylcysteine on *Panagrellus redivivus*

In the present experiments, N-acetylcysteine successfully reduced the toxic effects of ZnO particles on the nematode *Panagrellus redidivus*. So far, the mitigating effect of NAC on nano-metal oxides has been demonstrated mainly in human cell testing. The main purpose of these studies is to reduce unwanted effects of ZnO in cancer research and other human applications. In addition, studies have been researching the sensitivity of water flea (*Daphnia magna*), where NAC also mitigated the toxicity. For nematodes, the combination of ZnO NPs and NAC has not been studied so far, but the toxic effects of other nanomaterials, such as Al_2O_3 and TiO_2, have been successfully mitigated with NAC.

No specific reference has been found in the scientific literature regarding the effect of N-acetylcysteine on the dissolution of Zn, but the chelating properties of the material have been described in several studies. Solubility values in the present study were significantly higher when NAC was added. This is due to the fact that the total Zn content was measured, so that Zn particles held in complex by the NAC were also included. While from the two tested pure ZnO particles approximately the same amount of Zn dissolved, after addition of NAC significantly lower values were obtained from the larger particle size ZnO. The NAC was able to bind ions in a higher proportion from 15nm ZnO and thereby has a larger toxicity mitigation effect. This is presumably due to the fact that the two materials had different surface charge densities, distributions and electrical potentials due to their different size and morphology. Thus, N-acetylcysteine was more likely to complex with particles with substantially more opposite charges. In the present experiment, the NAC-treated control groups always had a higher mortality rate than the Milli-Q water control groups. This is due to the fact that NAC alone can be marginally toxic, especially at higher concentrations. My experiments have shown that N-acetylcysteine reduces the toxicity of ZnO NPs particles on *Panagrellus redivivus*.

5.1.4. Evaluation of reactive oxygen radical generation method

During the method development managed to modify the reactive oxygen radical measurements described by two published protocols so it could be usable and reproducible with the test species and indicator material used in the present study. Without the improvements, the method could not have been used. Subsequently, we applied the refined method successfully several times. In the future, it can be used to measure the induced intracellular reactive oxygen species with APF indicator on the *Panagrellus redivivus* test species.

5.2. Evaluation of particle size-dependent toxic effects and the underlying mechanisms

Scanning electron microscopy revealed that the 15 and 140 nm particle sizes reported by the manufacturer actually correspond to 59 ± 31 and 174 ± 138 nm. In addition, both materials contain nano and bulk particles, although not more than 50% of the all particle. Therefore 15 nm is still in the nano and 140 nm still in the bulk size range. Nevertheless, these properties probably influenced the results of the present experiments. It is hypothesized that if the nominal particle sizes were true, the smaller particle size would have been more toxic in the individual studies (without the influence of the test medium and the mitigating agent), as opposed to the relatively

similar toxic effects that experienced in this case. However, it cannot be ruled out that the use of the soil solution, the soil medium and the addition of N-acetylcysteine would change the results from those of the present studies. In addition, in my opinion, even stronger synergistic increase could have been experienced in the case of the mixture if the given sizes were the real ones.

5.2.1. Studies on test media and routes of exposure

Several studies pointed out no difference in the toxicity of ZnO NPs and bulk ZnO on soil organisms, while others have demonstrated the ZnO NPs to be more toxic than the bulk ZnO. The main reason for the greater toxicity of ZnO NPs could be that the smaller particle-sized ZnO dissolves more readily in water than the bulk form. In contrast, the 140nm ZnO I used showed higher toxicity in the presence of soil solution (*P. redidivus*) and artificial soil (*F. candida*) than its smaller particle-sized counterpart. Conversely, in our tests, the 140 nm ZnO proved to be significantly more toxic in soil solution with *P. redivivus* and artificial soil with *F. candida*. It seems plausible that the ZnO of the smaller particle size was better aggregated in the presence of soil and this resulted in lower toxicity. This theory is supported by the measured dissolution, as there were less free zinc ions in the case of 15 nm ZnO in soil solution after 48 h. However, in the Milli-Q aqueous medium, the dissolution of 15 nm ZnO was nearly twice that of the larger particulate. The tested ZnO particles have different shapes (spherical for 15 nm ZnO and irregular for 140 nm ZnO), which may also affect the toxicity of the particles, with 140 nm ZnO being more toxic. Based on these findings, the higher particle size ZnO (140 nm) had higher toxicity in the soil solution for *Panagrellus redidivus* and in artificial soil medium for *Folsomia candida* than the smaller particle size ZnO (15 nm).

5.2.2. Testing reactive oxygen radical production

Concentration-dependent production of reactive oxygen species is generally detected in ZnO NPs assays. Compared to other metal oxides, H_2O_2 production is medium dependent, but superoxide production was always highest in the case of ZnO NPs. In most cases in the literature comparisons of nano- and bulk ZnO were found to have a clearly visible particle size-dependent effect; ZnO NPs induced higher ROS generation. In the present experiments, the highest amount produced after 3 hours of exposure time was in the mixture and the smallest was in the 140 nm ZnO. When NAC was added, reactive oxygen species were produced to a much greater extent than independently when compared to the Milli-Q water control group. In the literature, ROS production is generally reduced by NAC. It is hypothesized that NAC-induced GSH production has not yet begun during the 3-hour ROS exposure, so the decrease was not experienced.

5.2.3. In the case of N-acetylcystein addition

Particle size-dependent mitigation is less elaborated in literature, since bulk controls were lacking in most cases. A study on this topic compared the effects of two nano-sized ZnO particles (18.5 ± 1.2 nm and 47.1 ± 5.1 nm ZnO) on the human neuroblastoma SHSY5Y cell line. When tested alone, the smaller particle size was more toxic, and the addition of NAC reduced the toxicity of both particle sizes. Mitigation was strongest at concentrations below 40 mg/l, above which a reduced effect was observed and also a slight difference in toxicity between the two particle sizes. In the case of the higher particle size, the mitigation was smaller, similarly to the present studies, where milder effect was observed in the case of 140 nm ZnO. In addition, similarly to the experiments in this paper, the study compared the effects of ZnO NPs with $ZnCl_2$. Although mitigation was observed in this case at two lower concentrations (122.9 μM and 245.7 μM $ZnCl_2$), above this, the cell survival rate decreased below 10% even with the addition of NAC. Therefore NAC had a much weaker effect on $ZnCl_2$ than on the nanoforms, similar to the present results. In

addition, the concentrations of $ZnCl_2$ used in these experiments represented the amount of dissolved Zn ions present in the test system. It can be clearly seen that behind the toxic effects of ZnO particles could be additional properties not just the dissolved ions toxicity. These results correlate with other studies where it has been shown that the amount of Zn^{2+} dissolved and the amount of ROS generated could not induce the degree of toxicity that was observed. Thus, it is believed that additional toxicity factors must be present to achieve the observed effects. According to the present experiments, NAC has a lower effect on ionic toxicity. In addition, the two substances I used have different toxicity mechanisms, so the larger particle size form proved to be more toxic.

5.2.4. In the case of the interaction between ZnO nanoparticles

By mixing two different nanomaterials, other works have also shown an increase in synergistic toxicity, e.g. ZnO NP + AG NP; Au NP+ Pt NP and Ag NP + TiO_2 NP. In some cases, however, a reduction in negative effects has been observed. In the literature, a reduction in toxicity was observed when nanoparticulate ZnO and TiO_2 were mixed, and this effect was explained by a Zn^{2+} adsorbed on the TiO_2 surface and thus became less available for the test animals. In the present studies, the binary mixture of two different particle sizes ZnO NPs showed a strong increase in toxicity. The SEM images confirm that both materials are present in the new mixture, so that a new ZnO with an average particle size between the two other materials (130 ± 118 nm), with both spherical and irregular particles, has been generated. In the case of the mixture, Zn dissolution were significantly and ROS production were marginally higher than in the case of the substances alone. The increase in toxicity was also observed with the addition of N-acetylcysteine, the lowest mitigating effect was observed in the case of the mixture. According to experiments with NAC, the antioxidant has less influence on the toxic effects caused by dissolved ions. The measured dissolved ion amount was 1.5 times higher in the mixture than in the two substances individually. In addition, N-acetylcysteine was able to bind the least amount from this material (Mixture <140 nm <15 nm). Particle distributions also show that ~ 30% of the particles can be found in the 140 nm ZnO are in the nano size range (37-97 nm, most often 37 nm), so the ratio of 30 nm has increased due to the mixing of the two materials in the mixture. In addition, larger particles are believed to have a dispersing effect on the test system. In the mixture, the particles aggregated more with each other due to the different charge distribution than with particles of their own size group. As a result, small particles aggregated on the surface of 140 nm ZnO particles fixed the large surface, increasing dissolution, reactivity, and thus toxicity. Therefore, by mixing the particles, the different negative effects of the two materials (15 nm - particle size, 140 nm - irregular morphology) reinforce each other, which may be the reason for the stronger toxic effects.

5.3. Summary of conclusions

Generally speaking, all two groups of soil organisms were sensitive to 15 and 140 nm ZnO treatment. The more sensitive species was the bacterivore nematode, *Panagrellus redivivus* in Milli-Q water test medium. Studies have shown that various media (Milli-Q water, soil solution), physical and biological properties of test soils and exposure routes (oral, dermal and oral) also influence the toxic effects of the test substances. In addition, N-acetylcysteine is able to mitigate the toxic effects of both studied particle sizes.

With the modified method, reactive oxygen species production was induced by all used material. In the case when testing the pure substances the highest was by the mixture and the lowest was by the 140nm ZnO. With the addition of NAC, due to the low toxicity of the substance itself all values were somewhat higher than with the individually tested compounds. Higher exposure times are required for the assay to be able to exert its effect.

From the two particle sizes applied, 140 nm ZnO was found to be more toxic several times (soil solution - *P. redidivus*; artificial soil - *F. candida*; mitigation studies - *P. redivivus*). This can be explained by the faster and stronger binding of 15 nm ZnO particles to soil particles, morphological differences, difference in charge distribution, and the fact that N-acetylcysteine was less able to make complexes with this material than with smaller particle sized one. This is probably also due to the morphological differences.

Besides, the NAC had less mitigation on the toxic effect of zinc ions. Therefore, it was possibly one of the causes to not observed statistically verifiable mitigating effect in the case of the mixture of the two substances, where the solubility was significantly higher. The $ZnCl_2$ concentrations I used represented the amount of dissolved Zn ions present in the test system, thus demonstrating that not only the ions and the ROS produced by the dissolution stand behind the toxic effects of the ZnO NPs, but other particle size-dependent toxic effects are also responsible for it.

When the two materials were applied in binary mixtures, the toxic effects increased significantly. Besides, the dissolved zinc content and the ROS generation is also increased. It is assumed that the chemical and physical properties of the materials (several smaller particles - higher bioavailability, increased toxicity from a fixed, large surface area, morphological aspects) have been mutually reinforcing to form a much more reactive mixture that is more easily absorbed by the *P. redivivus* test organism.

Studies have shown that ZnO alone can generate higher amounts of ROS and dissolved ions than 140 nm ZnO. On the other hand, if an additional test element neutralizing the toxic effects of 15 nm ZnO is added (eg soil, soil solution test medium, mitigating agent), 140 nm ZnO will be more toxic immediately. From this, it can be concluded that there is a third factor for this material, which is less influenced by soil particles and N-acetylcysteine. This is presumably due to the irregular particle morphology.

6. SCIENTIFIC PUBLICATIONS

6.1. Publications related to the topic of the thesis

Kiss, L. V.; Seres, A.; Hrács, K.; Nagy, P. I. (2015): The toxic effects of different particle sized zinc oxide on terrestrial springtail and nematode test organisms. Állattani Közlemények (Zoological Communications). 100 (1-2): 77-88.

Kiss, L. V.; Seres, A.; Hrács, K.; Nagy, P. I. (2016): The toxic effects of nanosized metal oxides on terrestrial microorganisms - A review. Agrokémia és Talajtan (Agrochemistry and Soil Science) 65: (1) 115–134

Kiss, L. V.; Hrács, K.; Nagy, P. I. .; Seres, A. (2018): Effects of Zinc Oxide Nanoparticles on *Panagrellus redivivus* (Nematoda) and *Folsomia candida* (Collembola) in Various Test Media. International Journal of Environmental Research. 12(2):233

Hrács, K., Sávoly, Z., Seres, A., **Kiss, L.V**., Papp, I.Z., Kukovecz, K., Záray, Gy., Nagy, P.I. (2018): Toxicity and uptake of nanoparticulate and bulk ZnO in nematodes with different life strategies. Ecotoxicology. 1-11.

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Kiss L. V., Nagy P.I., Seres A. (2017): Nanomaterials in soil life: Fearsome little ones. TermészetBúvár 72: (2) 10-12.

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6.2. Presentations related to the topic of the thesis

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Kiss, L. V.; Seres, A.; Nagy, P. I. (2018): Toxicity mitigation by N-acetylcysteine and photo-induced toxicity of zinc oxide nanoparticles on a bacterivor nematode: *Panagrellus redivivus* In: TOX'2018 conference (2018) pp. 52-52. Paper: C1-2 , 1 p.

Kiss, L. V. (2019): The effect of UV light and Toxicity mitigation of zinc oxide nanoparticles. ÚNKP Conference.

6.3. Posters related to the topic of the thesis

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Kiss, L. V; Hrács, K.; Nagy, P. I. .; Seres, A. (2016): The toxic effects of different particle sized zinc oxide on terrestrial springtail and nematode test organisms. XVII International Colloquium on Soil Zoology, Nara, August 22th-26th 2016.

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Kiss L. V., Hyodo F., Nagy P.I., Seres A. (2018): Differently sized zinc oxide nanoparticles effects on microbial biomass. SETAC Europe 28th Annual Meeting, Róma, Olaszország. 2018.05.13-17.

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