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Effect of zink oxyde nanoparticles on the test function of water organisms of different trophic levels

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Abstract. The toxicity of zinc oxide nanoparticles (nZnO) with particle size $\Delta_{50} = 20$ nm was evaluated according to the degree of toxicity of the aqueous disperse system (DS) with biological testing methods using a set of test organisms representing the major trophic levels. We observed the influence of the concentration degree of nZnO on toxic effects level on the fluorescence of the bacterial biosensor "Ekolyum-13", the chemotactic response of ciliates *Paramecium caudatum*, the rate of growth of unicellular algae *Chlorella vulgaris Bayer*, mortality of entomostracans *Daphnia magna Straus* and fish *Danio rerio*. The detected values of L(E)C₅₀ are: for biosensor "Ekolyum-13" - 0.30 mg/L, for ciliates *Paramecium caudatum* - 0.14 mg/L, for *Chlorella vulgaris Bayer* - 0.17 mg/L and for *Daphnia magna Straus* - 0.52 mg/L. No toxicity of nZnO was detected in relation to fish *Danio rerio*, L(E)C₅₀ > 100 mg/L. In assessing the maximum effect of nZnO according to GHS and EU Directive 93/67/EEC, it is assigned to dangerous substances with a high degree of toxicity "Acute toxicity 1".

1. Introduction

Development of nanotechnology, growing production volumes of fine disperse materials (FDM), the exponential growth in the number of products containing engineered nanoparticles (NP), on the one hand, opens up new possibilities of using FDM in biomedicine, pharmacology, food production, in addressing environmental and agricultural issues. On the other hand, the unique properties of FDM may carry a potential risk to the environment.

The need to determine the safety of FDM, approved by regulatory documents of Russia and OECD, ISO, EU and the Environmental Protection Agency [1, 2], arises from the large volumes of production and a wide range of application of FDM.

Despite the fact that currently the volume of industrial production of various NP is more than 100,000 tons a year, studies on the safety of nanotechnologies and nanoproducts are scattered, fragmentary, incomplete and significantly far behind the FDM production rate growth and expansion of their scope of usage. To date, most of the published papers are devoted to the study of biological effects of the most popular metal oxides NP. According to Bondarenko et. al. [3] among metal oxides NP, the NP of zinc oxide (nZnO) hold the third place in terms of volume of production (nSiO₂ - 55,000 tons/year, nTiO₂ - 3000 t/year, nZnO - 550 tons/year). The wide range of applications of nZnO in the field of cosmetology, medicine, materials science [4, 5, 6, 7] is due to its anti-bacterial, surfactant and catalytic properties [8, 9]. The presence of biological effects when NP contact with representatives of the biota makes the research on the biological effects of FDM, identifying risks to the environment and ensuring biosafety as a necessary component in the development of



nanotechnology a priority. This necessity is determined by the growth of consumer goods on the basis of GMR, including those on the basis of nZnO, which will inevitably lead to their release into the environment and as a result in aquatic ecosystems - the ultimate receiving environment.

It is an acute problem that there is still no single unified analytical system that could provide a reliable risk assessment of NP for wildlife objects.

As a result of earlier (in 2008-2013) target programs and research studies we have developed a biological model and a series of biotests for ecotoxicity assessment [10]. An essential component of the algorithm for estimating toxicity we developed is the expanded core set of test organisms (representatives of the main trophic levels), which allows us to study not only of the FDM biocidal action, but also changes in the test - functions of living aquatic organisms.

2. Materials and methods

nZnO dispersed system (DS) with an initial concentration of 50 mg/l was obtained by laser ablation [11]. According to BET analysis and transmission electron microscopy the average particle size was $\Delta_{50} = 20$ nm.

nZnO toxicity study was conducted using the test organisms representing the main trophic levels of ecosystems: luminescent bacteria on the basis of genetically engineered strains of *Eshcherichia coli* (biosensor "Ekolyum-13") was used for studying bioluminescence change [10]; unicelled animals *Paramecium caudatum* - for the chemotactic response [10]; unicellular algae - for the optical density of the test culture algae *Chlorella vulgaris Beijer* [10]; entomostracans *Daphnia magna Straus* - in terms of mortality rate [10]; *Danio rerio* fish - also in terms of mortality rate [12]. The research design is similar to the described in [13, 14].

nZnO DS was formed according to the method we developed [15]. The study used nZnO DS at concentrations of 0.0001, 0.001, 0.01, 0.1, 1.0, 5.0 and 10.0 mg/l. Right before biotesting nZnO DS was sonicated with ultra sound (30 W/L) for 5 minutes for redispersion. Evaluation of toxicity of the analysed nZnO DS was carried out in accordance with the criteria prescribed in the certified techniques [16].

Statistical data processing was executed in Excel 2010. Probit analysis was used to calculate the values of $L(E)C_{10}$ (NOEC), $L(E)C_{20}$ and $L(E)C_{50}$. Work was conducted with the application of metrologically gauged instruments: Autolumat LB953 (Germany), «Shimadzu» RF-5301 PC spectrofluorimeter (Japan), IPS-03 photoelectric colorimeter (Russia), Biotester-2 concentration meter (Russia) and diffractometer «Shimadzu» XRD 6000 (Japan).

3. Results

Studies revealed that introduction of a low concentration of nZnO particles ($S \leq 0.001$ mg/l) resulted in suppression of the luminescence of the biosensor within acceptable values not exceeding 20 %. The toxicity index of nZnO DS for the tested range of concentrations was calculated according to the degree of luminescence decrease (figure 1). nZnO DS with particle concentration of 0.01 mg/l had a toxic effect on the biosensor and the toxicity index was slightly higher than the acceptable level ($I = 25.25 \pm 3.60$ %). Further increase of concentration of nZnO particles amplifies DS toxicity: when $C = 0.1$ mg/l ($I = 41.36 \pm 3.03$ %) and $C = 1.0$ mg/l ($I = 51.72 \pm 3.78$ %) the suppression of the biosensor luminescence is doubled. Further increase of nZnO concentration to 5 mg/l causes luminescence inhibition up to 59.84 ± 0.46 %. Almost complete suppression of biosensor luminescence was observed with nZnO DS concentration of 10 mg/l ($I = 93.75 \pm 6.48$ %).

When exposing *Paramecium caudatum* in nZnO DS in concentrations ranging from 0.0001 mg/l to 0.1 mg/l the index of toxicity did not exceed the acceptable range ($I = 22.5 \pm 1.4$ %, $I = 1.1 \pm 0.1$ %, $I = 9.4 \pm 2.6$ % and $I = 9.6 \pm 0.8$ %) (Figure 1). nZnO DS concentrations with 1.0, 5.0, and 10.0 mg/l for 30 minutes reduced the yield of *Paramecium caudatum* in the test medium to almost complete suppression of positive chemotaxis ($I = 93.5 \pm 0.5$ %; $I = 94.4 \pm 1.0$ % and $I = 85.0 \pm 0.9$ %).

When cultivating *Chlorella v. B.* in nZnO DS with concentrations of 0.1, 0.01, 0.001 and 0.0001 mg/l the toxicity index did not exceed the acceptable range (figure 1). nZnO DS concentration of 1.0

mg/l had more pronounced toxic effect on the growth of chlorella cells. The toxicity index at that significantly exceeded the acceptable range ($I=63.5\pm 0.73\%$). When chlorella was exposed to nZnO DS concentration of 5.0 mg/l and 10.0 mg/l it almost completely inhibited the growth of *Chlorella v.B.* The toxicity index was $I=97.3\pm 0.56\%$ and $I=98.4\pm 0.89\%$ respectively (figure 1).

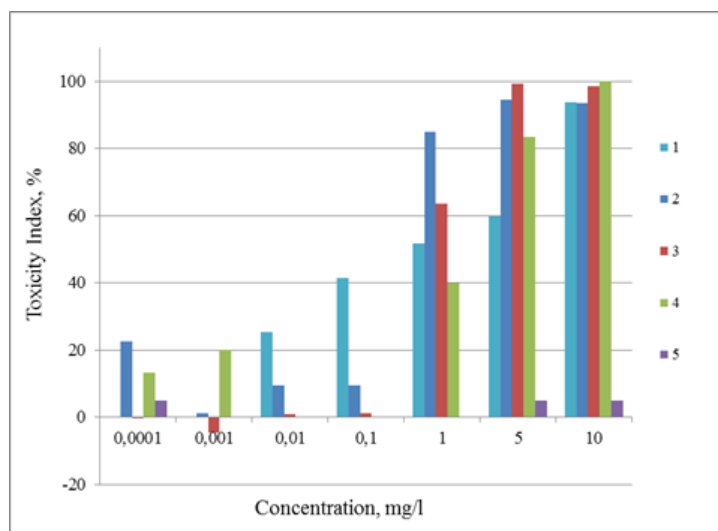


Figure 1. Index of the nZnO DS toxicity with a particle size of 20 nm for various test organisms (1 - «Ekolyum-13», 2 - *Paramecium caudatum*, 3 - *Chlorella vulgaris* Beijer, 4 - *Daphnia magna* Straus, 5 - *Danio rerio*)

When *Daphnia* was cultivated in DS nZnO with concentrations of 0.1 mg/l, 0.01 mg/l, 0.001 mg/l, 0.0001 mg/l for 48 hours, the mortality of these crustacean changed within the range of acceptable values from 0 to 20 % (figure 1). nZnO DS with concentration of 1.0 mg/l caused a high mortality rate of *Daphnia magna* Straus ($I=40.0\pm 4.1\%$), which is rated as not providing acute toxic effects, but having a fairly pronounced biological effects. Further increase of the concentration of nZnO DS ($C=5$ mg/l) resulted in an increase in mortality of crustaceans ($I=83.3\pm 2.4\%$). nZnO DS with concentration of 10 mg/l caused 100 % mortality of *Daphnia magna* Straus (figure 1).

nZnO DS (20 nm) in the test concentrations from 0.0001 mg/l to 10.0 mg/l did not cause mortality in fish.

Ecotoxicological parameters NOEC ($L(E)C_{10}$), ($L(E)C_{20}$ and $L(E)C_{50}$) of nZnO DS have been defined for each test organism. When formulating the expert opinion on ecotoxicity of the test sample we took into account the principle of assessment according to the most pronounced reactions reflected in the regulations [16, 17].

Observations proved that nZnO DS does not have biological effects on the biosensor with the concentration of up to 0.028 mg/l (NOEC). Toxic effects began to show with the increase in concentrations of up to 0.063 mg/l ($L(E)C_{20}$); $L(E)C_{50} = 0.30$ mg/l.

nZnO DS concentrations ranging less than 0.002 mg/l (NOEC) caused no biological effects in infusoria *Paramecium caudatum*. The toxic effect was observed starting from concentrations of 0.008 mg/l ($L(E)C_{20}$); $L(E)C_{50} = 0.14$ mg/l.

nZnO DS concentrations ranging less 0.013 mg/l (NOEC) caused no biological effects in the algae *Chlorella vulgaris* Bayer. The toxic effect was observed starting from concentrations of 0.059 mg/l ($L(E)C_{30}$); $L(E)C_{50} = 0.17$ mg/l.

nZnO DS concentrations ranging less 0.007 mg/l (NOEC) caused no biological effects in *Daphnia magna* Straus. nZnO DS toxicity manifested itself starting with a concentration of 0.031 mg/l ($L(E)C_{20}$); $L(E)C_{50} = 0.518$ mg/l.

DS nZnO proved to be not toxic for fish *Danio rerio*; $L(E)C_{50} > 100$ mg/l.

Basing on the data of ecotoxicological assessment conducted according to the criteria of regulatory documents [16, 17] we carried out an integrated assessment of nZnO toxicity. The distribution of test

organisms according to the "toxicity zones" (figure 2) can be represented more clearly using the scheme proposed by Bondarenko et. al. [3].

In contrast to these authors, the figure presents the results of the experiments we conducted according to a unified algorithm using a core set of test organisms, which was looking into both biocidal properties of FDM and their impact on test functions of living organisms.

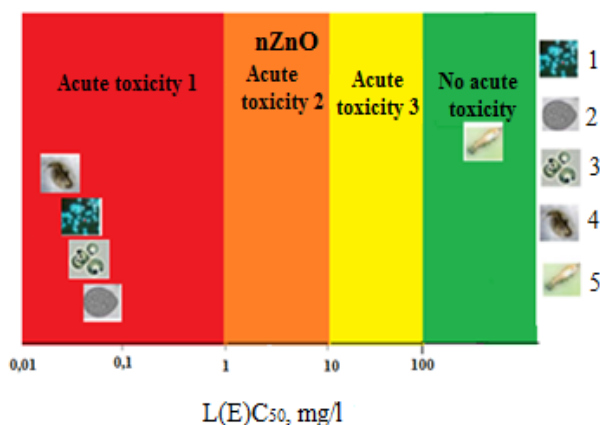


Figure 2. Classification of nZnO toxicity: 1 - «Ekolyum-13», 2 - *Paramecium caudatum*, 3 - *Chlorella vulgaris* Beijer, 4 - *Daphnia magna* Straus, 5 - *Danio rerio*

4. Discussion

The lack of a single unified analytical system, that provides reliable risk assessment of FDM to wildlife, does not allow us to properly compare the obtained results with the data available in the literature, most of which were obtained for 2, in a few cases for 3, test organisms. The authors of publications that attempt at presenting an integrated assessment of FDM toxicity, as a rule, do not take into account the size of particles, the specificity of NP DS preparation method and NP behaviour in cultured and natural media with varying degrees of mineralization, which significantly affect the assessment of toxicity of NP under study in the natural medium.

Bondarenko O. et. al. [3] notes in his review that the greatest interest lies in studying the toxicity of the three most popular types of NP: nAg, nCuO and nZnO. The number of publications devoted to the study of nZnO toxicity in recent years has grown up to 4,000.

Our results are mostly consistent with the published data on the damaging effects of nZnO on certain elements of ecosystem.

Pakrashia S. et. al. point out the damaging influence of nZnO on aquatic types of bacteria (*Vibrio fischeri*) depending on its concentration [18]. The toxic effect of nZnO sized 50-70 nm on ciliates *Tetrahymena thermophila*, according to Mortimer et. al. [19], may be associated with the transition of NP into the ionic form and nZnO aggregation.

Pendashte H. et. al. [20] investigated the effect of nZnO sized 20 nm on the change of *Chlorella v.* and *Scenedesmus d.* cell density. The inhibitory effect on *Chlorella v.* cell growth manifested itself as early as 24 hours after the start of the experiment: the number of cells decreased with the increasing of nZnO concentrations. The most significant decrease in cell growth was noted at a concentration of 1.0 mg/l. The authors found that the degree of toxicity of nZnO is different for species: nZnO was more toxic for *Chlorella v.*

Lopes S. et. al. [21] estimated the toxic effect of nZnO with a particle sizes of 30 nm and 80-100 nm on the survival rate of *Daphnia magna* Straus. The values of L(E)C₅₀ for nZnO were 1.02±0.24 mg/l and 1.10±0.05 mg/l, respectively, which is a sign of acute toxicity of the tested NP. The present study did not indicate dependence between toxicity and the initial particle size. The authors suggest that during the experiment, due to nZnO sedimentation, *Daphnia* were exposed not to individual particles, but agglomerates, which were likely to modify the toxicity. Authors believe that nZnO can act as an additional source of Zn ions being released into the environment, causing changes in daphnia

reproductive function. Dependence of toxicity on the NP size was established by Liu et. al. [22] in their research, which studied the effect of nZnO (30 nm, 50 nm) on the survival and reproduction of *Daphnia magna Straus* and the development of *Danio rerio* fish embryos. The authors found that nZnO sized 30 nm is more toxic than nZnO with 50 nm particle size. Researchers believe that nZnO (30 nm), unlike nZnO (50 nm), easily penetrates into the lymphatic, digestive and blood circulatory systems, thereby causing a greater toxic effect. *Danio rerio*, exposed to nZnO sized 30 nm and 50 nm, produced less embryos with the increase of concentration from 1.0 mg/l to 25.0 mg/l. Furthermore, not a single embryo appeared after applying nZnO of the same sizes in concentrations ranging from 50.0 mg/l to 100.0 mg/l [22].

The conducted research allowed us to determine the "limiting elements" of the ecosystem stability to the nZnO environment contamination. The degree of NP effect depends on the type of a test organism and the concentration of NP. The most sensitive to nZnO are the protozoaires *Paramecium caudatum* (L(E)C₅₀ = 0.14 mg/l) and unicellular algae *Chlorella vulgaris Bayer* (L(E)C₅₀ = 0.17 mg/l).

5. Conclusion

It has been determined that nZnO, obtained by laser ablation, with a particle size of $\Delta 50 = 20$ nm, belongs to dangerous substances with a high degree of toxicity "Acute toxicity 1".

It has also been found that the acceptable/safe level of the tested NP does not exceed the concentration of 0.001 mg/l. Higher concentrations of nZnO put at risk some representatives of the trophic chain, which causes damage to the integrity of the natural biocenosis and its ability to recover.

The increasing of concentrations of nZnO to 5.0 mg/l leads to significant toxic damage to test organisms (excluding fish).

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