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Comparative Evaluation of Acute Toxicity of Nanoparticles of Zinc, Copper and their Nanosystems using *Stylonychia mytilus*

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ABSTRACT

Nanotechnology has revolutionized the world by introducing a unique class of materials and consumer goods in many areas. This led to the production of new materials and devices. Nanotechnology has become an important priority in many countries. Despite its unique advantages and applications in everyday life and industry, the use of materials with nanometer dimensions raised a question of their safety for consumers and the environment[1].The rapid propagation of various nanomaterials is a dilemma for regulatory authorities in relation to their hazard identification[2]. Recently, it has been written a lot about the urgent need to develop rules for nanomaterials. Such rules are needed for legislators to protect people from potentially adverse effects. As for manufacturers these rules are necessary in order to prove that nanoproducts must be produced carefully and with caution to avoid possible negative effects. However, it is turned out to be difficult to develop such rules[3]. At present, many efforts are made to use relatively simple nano-structured materials, such as metal oxide nanoparticles and carbon nanotubes in the content of high-strength materials, self-cleaning surfaces and stain-resistant textiles and for energy storage and conversion[4]. The study of more complex nanomaterials will lead to the use of these data in medical diagnostics and treatment, and modern electronics[5].Within the past decade nanomaterials are used on a new commercial scale, it increases risks of possible toxic effects of nanoparticles that enter the environment because their potential toxicity for aquatic organisms is high[6]. Toxic effect of nanoparticles for aquatic ecosystems is associated with their physical and chemical properties and their action in aqueous media where dissolution, aggregation and agglomeration may occur[7]. Despite the fact that the production of these particles increased considerably in recent years, there are little data on their toxicity. Results of toxicity differ mainly due to the different methodology[8]. Different biological tests were discussed; they have been successfully used to assess the environmental impact of pollutants on invertebrates, algae and bacteria. Now they are more often used for risk assessment of nanoparticles on different levels of the aquatic food web[9].Environmental risks and methodological approaches for testing the toxicity of nanomaterials using aquatic organisms were considered in recent publications[10]. For example, the toxicity of CuO and ZnO nanoparticles was assessed using a simplified model of the aquatic food chain in order to determine the bioaccumulation of toxic effects and particle transport through the trophic levels[11].The experiment of using bacteria, protozoa, yeast, rotifer, algae, nematodes, crustaceans, fish, and amphibians as test objects in wastewater toxicological studies was described[12]. Only individual effects of nanoparticles were studied in most works. Despite the implementation of these data as a tool for assessing risks to the aquatic environment, there are still not enough data to fully understand the toxic effects of nanoparticles on aquatic organisms[13]

Key words: *Stylonychia mytilus*, nanoparticles, zinc, copper, cell growth inhibition, cytotoxicity

INTRODUCTION

Objectives

The aim of this study was to determine the toxicity of different doses of nanoparticles of zinc, copper and their compounds and the use of *Stylonychia mytilus* as a model organism.

MATERIALS AND METHODS

Fresh water *Stylonychia mytilus* (wild strain) was utilised in the experiments during its exponential growth phase. The tested functions were survival and quantity (biomass). The cells were cultured on the medium of Lozina-Lozinskii with the addition of yeast (*Saccharomyces cerevisiae*) into the growing medium: NaCl-0,1%; KCl-0,01%; $CaCl_2$-0,01%; $MgCl_2$-0,01%; $NaHCO_3$-0,02%. The concentrated medium was dilution with distilled water.

Cells at a stable growth rate were incubated at 20 ± 2°C. The particles were added to the cell culture for 24 hours. The quantity of cells was recorded after 1, 6, 12 and 24 hours. The amount of cells was determined utilising a light microscope (MT 5300L). The sensitivity of *S. mytilus* to the toxic particle was determined based on the time of the cell death, detected by a termination in movement of the protozoa together with cell membrane lysis. The amount of *S. mytilus* in 5 ml medium (without the addition of the particles) was utilised as a control for all experiments. The nanoparticles utilized in the study are described in table 1.

Nanoparticles preparation was conduct in isotonic solution with an ultrasonic disperser (f-35 kHz, N 300 W), by dispersing for 30 minutes. The size of the particles was determined by a Field Emission Scanning Electron *Microscope (JSM 740 IF)*. The concentration of the toxic particles varied between $6 \cdot 10^{-6}$ M and 3.2 M.

ANOVA statistical analysis was utilized and then using the Tukey test (SPSS version. 17.0). Differences were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

Analysis of the data revealed different effects of nanoparticles of zinc, copper and nanosystems simplest cell culture (Table 2).

Table 1: Characteristics of the utilised nanoparticles

Name	Size [nm]	Phase and chemical composition	Production method	Surface (m^2/g)	Producer
Zn	90	90%, the rest sorbing gases, zinc oxide and H2O	the electric explosion of wire in an argon atmosphere	5,34	«Advanced powder technologies», Russia
Cu	97	crystal copper 96,0 ± 4,5%, copper oxide - 4,0 ± 0,4%	high-temperature condensation with subsequent modification of oxygen	24	«Advanced powder technologies», Russia
CuZn(alloy)	65	60% copper and 40%zinc	the electric explosion of wire in an argon atmosphere	5-6	«Advanced powder technologies», Russia
Cu-Zn (mixture)	96,5	60% copper and 40%zinc	Gas-phase	10	«Advanced powder technologies», Russia

Table 2: Effects of nanoparticles of zinc, copper and their compounds in cell culture *S. mytilus*

Concentration,	Time of exposition: 1 hour				Time of exposition: 6 hour			
mM	Zn	Cu	CuZn (alloy)	Cu-Zn (mixture)	Zn	Cu	CuZn (alloy)	Cu-Zn (mixture)
3,2	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
1,6	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,8	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,4	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,2	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,1	LC50	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,05	LOEC	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,025	LOEC	Tox	Tox	Tox	LC50	Tox	Tox	Tox
0,0125	NOEC	Tox	Tox	Tox	LOEC	Tox	Tox	Tox
0,00625	NOEC	Tox	Tox	Tox	LOEC	Tox	Tox	Tox
0,003	NOEC	Tox	Tox	Tox	NOEC	Tox	Tox	Tox
0,0015	NOEC	Tox	Tox	Tox	NOEC	Tox	Tox	Tox
0,00078	NOEC	LC50	Tox	Tox	NOEC	Tox	Tox	Tox
0,00039	NOEC	LOEC	Tox	Tox	NOEC	LC50	Tox	Tox
0,00019	NOEC	LOEC	LC50	Tox	NOEC	LOEC	Tox	Tox
9õ10^{-5}	NOEC	LOEC	LOEC	Tox	NOEC	LOEC	LC50	Tox
4õ10^{-5}	NOEC	NOEC	LOEC	LC50	NOEC	LOEC	LOEC	Tox
2õ10^{-5}	NOEC	NOEC	LOEC	LOEC	NOEC	NOEC	LOEC	LC50
1õ10^{-5}	NOEC	NOEC	LOEC	LOEC	NOEC	NOEC	LOEC	LOEC
6õ10^{-6}	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
	Time of exposition: 12 hour				**Time of exposition: 24 hour**			
3,2	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
1,6	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,8	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,4	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,2	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,1	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,05	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,025	LC50	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,0125	LOEC	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,00625	LOEC	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,003	NOEC	Tox	Tox	Tox	Tox	Tox	Tox	Tox
0,0015	NOEC	Tox	Tox	Tox	LC50	Tox	Tox	Tox
0,00078	NOEC	Tox	Tox	Tox	LOEC	Tox	Tox	Tox
0,00039	NOEC	LC50	Tox	Tox	LOEC	Tox	Tox	Tox
0,00019	NOEC	LOEC	Tox	Tox	NOEC	Tox	Tox	Tox
9×10^{-5}	NOEC	LOEC	LC50	Tox	NOEC	LC50	Tox	Tox
4×10^{-5}	NOEC	LOEC	LOEC	Tox	NOEC	LOEC	Tox	Tox
2×10^{-5}	NOEC	NOEC	LOEC	LC50	NOEC	LOEC	LC50	Tox
1×10^{-5}	NOEC	NOEC	LOEC	LOEC	NOEC	LOEC	LOEC	Tox
6×10^{-6}	NOEC	NOEC	NOEC	NOEC	NOEC	LOEC	LOEC	LC50

Note. Tox – the concentration causing 0-39 % survival object; LC50 – the concentration causing 50% survival of the object; LOEC – the concentration causing 40-69 % survival object; NOEC – the concentration causing 70-100 % survival object. **(**P. Jackson, N. Raun Jacobsen, A. Baun, R. Birkedal, D. Kühnel, K. Alstrup Jensen, U. Vogel, H. Wallin Bioaccumulation and ecotoxicity of carbon nanotubes // Chemistry Central Journa, 2013. 7(1):154)

Toxic effect of initial nanoparticles and their nanosystems is specific and depends on the concentration and exposure time of the cells.

The object of the study was to compare the nanoparticles of copper, zinc and their composites, and subsequently determine the maximum toxic effect. In the course of the experiments it was found that nanoparticles of zinc (Zn), copper (Cu) and alloy (CuZn), mixture (Cu-Zn) have a similar effect on the cell of the test organism.

Table 3 shows the morphological changes in the cells (flocculation, rupture, atrophy), depending on the length of incubation, cell culture *Stylonychia mytilus* a toxicant.

Moreover, the localization of nanoparticles in the cells was dependent on the incubation time of *Stylonychia mytilus* and toxicant. Thus, peripheral distribution of the nanoparticles was observed in the cells after 1 and 6 hours of incubation. After 12 hours of incubation uniform distribution of nanoparticles in the cell was observed.

The results demonstrate no effect of the nanoparticles in the first hour of incubation and complete destruction of the membrane beginning from 6 hours of incubation. During the evaluation of time periods it was registered that the maximum toxic effect of nanoparticles on *S. mytilus* is observed after 24 hours of incubation.

The analysis of concentration effects has shown that Zn nanoparticles are less toxic (LC50

Table 3: Morphological changes of cells *S. mytilus* on the background of toxic effects of nanoparticles

Time of incubation, h	Cell morphology			
	Zn	**Cu**	**CuZn (alloy)**	**Cu-Zn (mixture)**
1	Normal	Normal, flocculation	Normal, flocculation	Normal, flocculation
2	Flocculation	Flocculation, rupture	Flocculation	Flocculation
6	Flocculation, rupture	Flocculation, rupture, atrophy	Flocculation, rupture	Flocculation, rupture
12	Flocculation, rupture	Rupture, atrophy	Rupture, atrophy	Rupture, atrophy
24	Flocculation, rupture,atrophy	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy

Table 4: Biological effects of nanoparticles on *Stylonychia mytilus*

Name	Concentration, mM			
	ToX	**LC50 Metals**	**LOEC**	**NOEC**
Cu	3,2 - 0,00019	9×10^{-5}	4×10^{-5} - 2×10^{-5}	1×10^{-5} - 6×10^{-6}
Zn	3,2 - 0,003	0,00015	0,00078 - 0,00039	0,00019 - 6×10^{-6}
CuZn (alloy)	3,2 - 4×10^{-5}	2×10^{-5}	1×10^{-5}- 6×10^{-6}	-
Cu-Zn (mixture)	3,2 - 2×10^{-5}	1×10^{-5}	6×10^{-6}	-

Tox – the concentration causing 0-39 % survival object; LC50 – the concentration causing 50% survival of the object; LOEC – the concentration causing 40-69 % survival object; NOEC – the concentration causing 70-100 % survival object.

0.0015M) than Cu nanoparticles and their composites (CuZn (alloy), Cu-Zn (mixture)) (9 * 10^{-5} LC50 and LC50 2 * 10^{-5} M, LC50 6 * 10^{-6} M) (Fig. 1).

Study of toxic effects of nanoparticles has showed that all test samples had various toxic effects on cells of the test culture (Table 4).

Cells of *S. mytilus* absorb nanoparticles of Zn, Cu, CuZn (alloy), Cu-Zn (mixture) and store them in digestive vacuoles. However, nanoparticles of Zn, Cu and their composites (CuZn (alloy), Cu-Zn (mixture)) inhibit the growth of cells and thus cause acute toxicity in the process of endocytosis and exocytosis. This fact inhibited cell growth even after 6 hour incubation and induced significant cytotoxic effects. Toxic effects of nanoparticles enhanced after 24 hours of incubation in connection with the inhibition of cell growth and increase of cytotoxicity

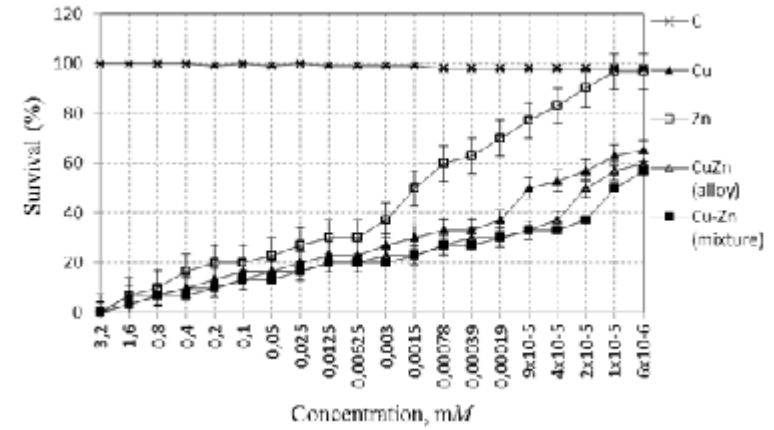

Fig. 1: Influence of metal nanoparticles with different concentration on survival of *Stylonychia mytilus*

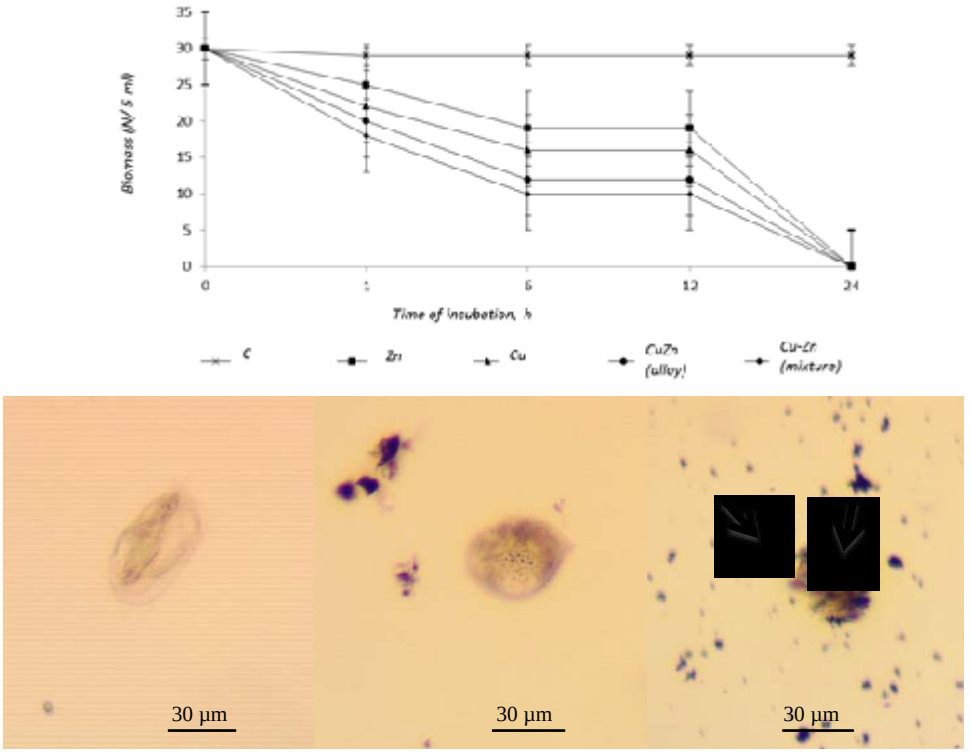

Fig. 2: Effects of forms of copper, zinc and their nanosystems in cell culture *S. mytilus*

of particles. After shorter time periods (1 hour) no changes were observed, the cells of protozoa remained active (Figure 2).

Growth inhibition and subsequent cell death are connected with the fact that, first of all, the adsorption of nanoparticles occurs on the cell surface, and then they link to the cell membrane and penetrate into cell. Studies have shown that nanoparticles are generally localized in digestive vacuoles (lysosomes), wherein the cell is trying to digest or secrete them into the environment.

The mechanism of nanoparticles penetrating into cell ("Trojan horse effect ") is widely described[14]. It is related to the fact that partially soluble nanoparticles of metal oxides penetrate into cells, but in a different ionic form. The oxidative stress is considerably higher than in the case of penetrating ions of the respective metals, the transfer is controlled[15]. Indeed, after metal oxide nanoparticles have entered the cell, they may dissolve more than structural units of cell releasing harmful concentrations of metal ions in the cytoplasm. For example, the release of cytotoxic amounts of Cd has been shown for CdSe quantum dots under physiological conditions[16]. Moreover, due to the high surface area, nanoparticles adsorb heavy metals, polycyclic aromatic hydrocarbons, quinoline. Mackevica, A. et al.[17] demonstrated that toxicity of phenanthrene for *Daphnia Magna* increased by 60% in the presence of C60 aggregates. Thus, nanoparticles act not only as transfer vectors in the environment, but they also promote the penetration of contaminants adsorbed in nanoparticles into the cells of organisms, potentiating the toxic effects[18].

Nanoparticles can traverse even the strongest biological barriers such as the blood-brain barrier[19]. For example, Oberdörster E. et al.[20, 21] have shown that influence of fullerenes (C60) caused oxidative damage in the brain of the fish. Despite this, until recently the nanoscale materials were considered as variations of technical material and, thus, individual registration was not required[22].

So far, the fate and behavior of nanoparticles in the aquatic environment is an important question in terms of their impact on the environment and potential toxicity. The comparative evaluation of the toxic effect of silicone dioxide (SiO_2) and titanium dioxide (TiO_2) on marine microalgae *Dunaliella tertiolecta* was perfomed. It was found that the change of the dose-response and population growth of *D. tertiolecta* algae depends on the presence of nanoparticles TiO_2. These particles influenced on 50% of the population. SiO_2 nanoparticles were less toxic than TiO_2 for *D. tertiolecta*. General toxic effect was established due to the contact between aggregates of nanoparticles and cell surfaces. SiO_2 nanoparticles have a direct effect on the integrity of the cell membrane on the fourth day of exposure, while TiO_2 nanoparticles exert their toxic effects in the first hours of exposure, resulting in mobile capture and agglomeration[23].

According to data obtained by Jagadeesh, E. et al. exposures of nanoparticles (iron oxide, cadmium sulfide nanocomposite and silver sulfide, cadmium sulfide and silver sulfide nanoparticles) caused lipid peroxidation and ROS, and suppressed the antioxidant defense system, such as catalase, glutathione reductase and superoxide dismutase of a model organism *Mougeotia sp*. The adsorption of nanoparticles on the surface of algae and membrane damage were confirmed through microscopic evaluation and increase of the protein content in the extracellular medium[24].

CONCLUSION

Thus, researches on nanoparticles safety are absolutely essential to support sustainable development of nanotechnology. Additional studies are needed because of the heterogeneity of the developing eco-toxicity of metal nanoparticles. The performed experiments indicate that specific biosensors (*Stylonychia mytilus*) that are highly sensitive to the toxic effect of metal nanoparticles (for example, Zn, Cu nanoparticles and CuZn (alloy), Cu-Zn (mixture) play a key role for the standardization of ecotoxicity of heavy metal nanoparticles.

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