Assessment of prospects for the use of unicellular hydrobionts as test objects in the study of biotoxicity of ultradispersed metal particles

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Abstract. Active development of nanomaterials in various areas of human activity leads to their intensive release into the environment. Nanomaterials in aquatic ecosystems are of particular concern, leading to changes in water quality and dangerous for representatives of aquabionocenosis - aquatic invertebrates, bioaccumulating technogenic nanoparticles. In this regard, studies on the selection of an optimal test object from representatives of hydrobionts, having the most adequate response to various, including toxic, effects of nanomaterials, with further prospect of determining the potential risks that arise when they enter aquatic biocenoses are relevant. Cultures of freshwater ciliate of Stylonychia mytilus (wild strain) in the phase of exponential growth and Paramecium caudatum were selected as the objects of the study. Survival was a determined parameter. 9 laboratory preparations of ultradispersed particles of metals (zinc, copper, ferrum, argentum, cobalt, titanium, aluminum, molybdenum, nickel) were used in studies. The analysis of the effects of ultradispersed metal particles revealed a negative biological effect on Paramecium caudatum and Stylonychia mytilus. Paramecium caudatum was the most sensitive to increasing concentrations of nanoparticles, as evidenced by the death of individuals after 10 minutes of contact. In turn, the death of Stylonychia mytilus was observed only at the 60th minute of exposure. Based on the above, Paramecium caudatum culture can be proposed as an express test object, giving a response at the 10th minute of the experiment. For studies assessing various types of toxicity that require a longer contact time, Stylonychia mytilus is a more suitable test object, since the effect of nanoparticles on the cell can be observed for 60-180 minutes or more.

1. Introduction
The growth of production and the successful implementation of nanomaterials in various areas of human activity raise concerns about the manifestation of specific nanoscale effects on living organisms. Rapid development of nanotechnology and industrial progress worsen the environment quality and lead to an increase in the diversity of pollutants, most of which ultimately end up in water bodies where they can be dissolved, aggregated and agglomerated [1].

One of the most likely ways for nanomaterials to enter the environment is wastewater [2]. This leads to deterioration of water properties both a resource for human activity and a habitat for aquatic organisms. Impairment of the vital activity of hydrobionts can change the functioning of the entire ecosystem, since they are the starting basis of trophic chains in which toxic substances can be accumulated from soil and sediment and then enter higher organisms.

Currently, various test objects are used to assess the effects of substances [3]: bacteria [4], animalcula [5, 6], sea weeds [7], worms [8, 9]. Correct selection of organisms for biotesting is one of the most important applied tasks in toxicology.

The most promising models for widespread use in various areas of biotesting are currently unicellular hydrobionts (animalcula). As test organisms, they have several advantages, for example, a large area of contact with the environment, due to which there is a rapid contact of the cell with the analyte. Their reactions to the objects of the study are direct and informative, with the reaction of the organism being a direct indicator of the biological effect of the studied substance. One of the most likely ways to use such test objects is the biotesting of nanomaterials.
The environment is expressed by a whole complex of changes: chemotaxis, response of ciliates to stress, and the environment, which allows us to assess the effect of the analyte [10]. A number of hydrobionts have a filtration type of food, which increases the likelihood of their accumulation of substances contained in the environment, which allows assessing the degree of their influence on the body [11].

Possible environmental risks and methodological approaches for testing the effects of nanomaterials using hydrobionts are discussed in recent publications [12]. The experience of using ciliates of Euglena, Vorticella and Stylonychia genera as test objects for toxicological studies of wastewater is described [13].

Also, representatives of free-living ciliary ciliates of Tetrahymena genus, in particular, the species of Tetrahymena pyriformis are quite successfully used to assess the toxicity and nutritional value of human and animals food [14]. Ciliates of Paramecium genus are also widely used in toxicological practice. The most well-known species – Paramecium caudatum, Paramecium putrinum, Paramecium aurelia, Paramecium multicaule make it possible to determine the dose-dependent effects of various pharmacological preparations, cosmetics, feed and food products by the chemotactic reaction of animalcule [15]. The biotesting results on Paramecium have a fairly high degree of correlation with the results obtained in experiments “in vivo” on warm-blooded animals [16].

In this regard, in order to monitor the environment state under the conditions of anthropogenic impact, in particular, studying the effects of ecotoxins and nanomaterials on living organisms, it is relevant to develop new and improve existing methods of bioindication and biotesting using unicellular hydrobionts.

The purpose of this work is to assess the toxic effect of ultradispersed particles and their oxides on the organism survival on the example of unicellular hydrobionts of Paramecium caudatum and Stylonychia mytilus.

2. Materials and methods

Cultures of freshwater ciliate of Stylonychia mytilus (wild strain) in the phase of active (exponential) growth and ciliate cell culture of Paramecium caudatum were selected as the objects of the study.

9 laboratory preparations of ultradispersed metal particles were used as research substances. The list included ultradispersed particles of zinc (Zn UDP), copper (Cu UDP), ferrum (Fe UDP), argentum (Ag UDP), cobalt (Co UDP), titanium (Ti UDP), aluminum (Al UDP), molybdenum (Mo UDP), nickel (Ni UDP). These ultradispersed particles passed through material science validation by the methods of JSM 7401F electron scanning and JEM-2000FX transmission microscopy (“JEOL”, Japan), as well as by the method of X-ray phase analysis on DRON-7 multifunctional diffractometer (RPE “Burevestnik”, Russia) and corresponded to physico-chemical characteristics described in Table 1.

Table 1. Characteristics of the studied ultradispersed metal particles

| UDP | Diameter, nm | Ssp.cov., m²/g | Z-potential, mV | Particle shape | Sample purity, % | Colour |
|-----|--------------|----------------|-----------------|----------------|-----------------|--------|
| Zn  | 98.0 ± 2.1   | 5.3            | -41.00 ± 0.81   | spherical      | 99.9            | dark grey |
| Cu  | 103.0 ± 2.0  | 12.0           | -31.00 ± 0.1    | spherical      | 96.0            | dark fulvous |
| Fe  | 100 ± 2.0    | 7.7            | 13.00 ± 0.5     | spherical      | 99.9            | fulvous |
| Co  | 100 ± 2.0    | 13.0           | 15.34 ± 0.5     | spherical      | 99.9            | grey |
| Ag  | 139 ± 16.0   | 6.5            | -37.00 ± 0.1    | spherical      | 99.9            | grey |
| Ti  | 80.0 ± 1.8   | 13.8           | -87.00 ± 0.96   | spherical      | 99.8            | grey |
| Al  | 90.0 ± 2.0   | 12.0           | -34.00 ± 0.65   | cubic          | 94.0            | grey |
| Mo  | 50.0 ± 1.5   | 14.0           | -43.00 ± 0.52   | spherical      | 99.7            | dark grey |
| Ni  | 70.0 ± 0.3   | 17.0           | -25.00 ± 0.50   | spherical      | 99.8            | dark grey |

Initial cell cultures of Stylonychia mytilus and Paramecium caudatum cultivated for seven days on Lozin-Lozinsky environment with the addition of yeast (Saccharomyces cerevisiae). To improve the quality of the study, it was first necessary to bring the culture into an exponential growth phase that was achieved by reseeding the culture in a Petri dish with the addition of feed three days before setting up the experiment [15].
The action of ultradispersed particles was studied in a wide range of concentrations (from 4×10^{-5} M to 0.5 M). The obtained samples of UDP in a volume of 200 µl were added to 1 row of cells of a 96-well microplate. 100 µl of distilled water was added to the remaining wells. Then a series of twofold dilutions was prepared from the initially obtained suspension [17]. Then, nanoparticle suspensions at the indicated concentrations were added to a pre-prepared 96-well microplate containing 10 individuals of the investigated unicellular hydrobionts in each well, captured using a Pasteur pipette. Control samples contained only 100 µl of distilled water and 10 individuals of the studied hydrobionts.

The sensitivity of animalcula cells culture to the action of nanoparticles was determined by a temporary indicator, an assessment of the number of dead cells, recorded by the absence of movement, which was accompanied by the integrity and lysosomes of the cell.

Counting the total number of cells in the environment containing ciliates was carried out using light microscopy. Intermediate calculation for Paramecium caudatum and Stylonychia mytilus was carried out after 10, 60, 180 minutes [18].

Also to assess toxic effects in cell cultures, the concentration of compounds was determined, which reduced cell viability by 50% (LC50). It is believed that LC50 index allows assessing the approximate levels of average lethal doses of chemical agents and going to MPC values [19].

Statistical processing was performed using ANOVA standard parameters; additionally Tukey criterion was used (SPSS pos. 17.0). Differences were considered statistically significant at P<0.05.

3. Impact assessment of ultradispersed metal particles on Paramecium caudatum and Stylonychia mytilus

During the study, it was found that Paramecium caudatum reacted to the effects of ultradispersed metal particles after only 10 minutes, which was expressed by a change in their numbers. Stylonychia mytilus turned out to be more resistant to ultradispersed particles and changes in their numbers were observed only after 60 minutes of exposure.

Thus, during the contact of ultradispersed metal particles with the studied Stylonychia mytilus test objects there was no change in their number at the 10th minute of the experiment and all used concentrations (Fig. 1). When using the second Paramecium caudatum test object, at 10th minutes of exposure, a change in their numbers was observed, this made it possible to fix the toxic effect of ultradispersed particles under study.

In the 60th minute of the experiment, the toxic effect of ultradispersed particles led to a decrease in viability and death of both target organisms. However, while studying the biological effects of titanium, copper, molybdenum and nickel nanoparticles, the survival values calculated for the 60th minute of the experiment for Stylonychia mytilus were similar to survival values of the 10th minute Paramecium caudatum contact, then Paramecium caudatum for cobalt and aluminum was more sensitive and the calculated survival values at 10th minute were lower than at 60th minute for Stylonychia mytilus.

The addition of ultradispersed particles of zinc, copper, iron and molybdenum resulted in the complete suppression of the vital activity and death of the test objects under study at the 180th minute of the experiment, both when using Stylonychia mytilus and Paramecium caudatum as test objects. In contrast, ultradispersed particles of titanium and aluminum showed weak toxicity to unicellular hydrobionts. So, in all the studied concentrations, death in both Stylonychia mytilus and Paramecium caudatum did not reach 50%, and the survival values were 70 and 65%, respectively, for titanium and 65% and 60%, respectively, for aluminum.

At the same time, at the 180th minute of contact Paramecium caudatum turned out to be more sensitive when assessing the toxicity of ultradispersed metal particles as compared with the second studied hydrobionts of Stylonychia mytilus. In fact, when adding ultradispersed argyment particles, LC50 for Paramecium caudatum was 4×10^{-5} M, and for Stylonychia mytilus – 0.0019 M. In this case, the survival of Stylonychia mytilus was 20 %, and Paramecium caudatum - 8 %.

When test organisms contacted with UDP of iron at the 180th minutes, LC50 for Paramecium caudatum was 4×10^{-5} M, and for Stylonychia mytilus - 0.0078 M. At the same time, the survival rate of Stylonychia mytilus was 37%, and Paramecium caudatum was 18%, which also indicates a faster response of Paramecium caudatum test object to the toxicant.
Figure 1. *Paramecium caudatum* and *Stylonychia mytilus* survival under the influence of ultradispersed particles of titanium (a), aluminum (b), cobalt (c), argentum (d), ferrum (e), nickel (f), molybdenum (g), copper (h), zinc (i) at the 10th, 60th and 180th minutes of contact
The action of ultradispersed nickel particles also made it possible to determine the concentrations of LC50 for animalcula at the 180th minute of the contact, which amounted to Paramecium caudatum 4×10⁻⁵ M and 9×10⁻⁵ M to Stylonychia mytilus. At the same time, almost 100% suppression of the vital activity of Paramecium caudatum was observed, the survival rate of Stylonychia mytilus was 27%.

UDP of molybdenum also turned out to be highly toxic to animalcula, as evidenced by the values of LC50, which at 180th minute amounted to Paramecium caudatum 4×10⁻⁵ M and to Stylonychia mytilus - 9×10⁻⁵ M. Thus, ultradispersed molybdenum particles caused almost 100% death of Paramecium caudatum at the 180th minute, while the survival value of Stylonychia mytilus was only 13%.

Ultradispersed particles of copper and zinc had a practically identical toxic effect on unicellular hydrobions. Thus, when adding UDP of copper LC50 at the 180th minute of contact was 4×10⁻⁵ M both for Paramecium caudatum, and Stylonychia mytilus. At the same time, Stylonychia mytilus survival was 12 %, and Paramecium caudatum - 8 %.

At the 180th minute of contact of ultradispersed zinc particles with Paramecium caudatum and Stylonychia mytilus cells LC50 was also 4×10⁻⁵ M for these test organisms and the survival was 11 % and 6 % for Stylonychia mytilus and Paramecium caudatum, respectively.

4. Conclusion
It has been revealed in the course of the research that entire range of ultradispersed particles has toxicity with respect to the used animalcula. In this case, the toxic effect of ultradispersed particles is specific and depends on the concentration and time of exposure to cells. [20]. As a proposed mechanism of their toxicity, it should be noted that the addition of ultradispersed particles leads to an increase in reactive oxygen species and, as a result, the development of oxidative stress, which can lead to cell death. [21-23]. Also, the mechanism of UDP toxicity is associated with the release of their ions into aquatic environment and their direct interaction with the cell membranes of the test objects, which causes an inhibitory effect on cellular functions [24-27].

Based on the obtained data, it is also possible to conclude that Paramecium caudatum is the most rapidly responding to increasing concentrations of nanoparticles, as evidenced by the death of individuals being observed after 10 minutes of contact. In turn, the cell death of Stylonychia mytilus occurred at a later exposure time, however, the severity of the effects was higher compared to Paramecium caudatum. Based on the above, Paramecium caudatum culture can be proposed as an express test object, giving a response at the 10th minute of the experiment. For studies assessing various types of toxicity that require a longer contact time, Stylonychia mytilus is a more suitable test object subject, since the effect of nanoparticles on the cell can be observed for 60-180 minutes or more.

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