Original article DOI: 10.29039/2413-5577-2023-1-124-136

Effect of Bicomponent ZnO-$ZnFe_2O_4$ Nanoparticles on Mediterranean Mussel (*Mytilus galloprovincialis*) Hemocytes under *in vitro* Conditions

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Abstract
The present work investigates the toxic effect of bicomponent ZnO-$ZnFe_2O_4$ nanoparticles, which are the main active component of the domestic antifouling coating, on marker indicators of Mediterranean mussel (*Mytilus galloprovincialis*) hemolymph cells (hemocytes) under *in vitro* experimental conditions. The following indicators were evaluated: mortality, cellular composition and production of reactive oxygen species. In the experiment, hemocytes were incubated for 1 and 2 hours in 1 mL of sterile seawater containing nanoparticles of different concentrations: 0.03, 0.3 and 3 mg/mL. The data were analyzed using the flowing cytometry. It was shown that ZnO-$ZnFe_2O_4$ nanoparticles had an effect on the cellular composition of the hemolymph: the proportion of agranulocytes decreased and hour exposure to 0.03 mg/mL nanoparticles reduced the level of production of reactive oxygen species by 2.5 times compared to the control ($p \leq 0.05$). Incubation of hemocytes with a maximum concentration of nanoparticles (3 mg/mL) led to cell death within 1 hour after exposure. No acute toxic effects on hemocytes with the use of 0.03 mg/mL and 0.3 mg/mL of zinc oxide and zinc ferrite nanoparticles were observed.

Keywords: nanoparticles, Mediterranean mussel, hemocytes, reactive oxygen species

Acknowledgements: the synthesis of nanoparticles (ZnO-$ZnFe_2O_4$) was carried out under RSF project no. 21-13-00498 “Environmentally safe and highly effective antifouling coatings based on bicomponent metal nanoparticles and their oxides”. Assessment of the toxicity of nanoparticles on the body of mussels (analysis of hemocyte parameters) was carried out at the expense of the state task of FRC IBSS No. 121102500161-4 “Patterns of the organization of the immune system of commercial hydrobionts and the study of the influence of environmental factors on the functioning of their protective systems”.

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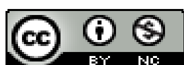

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For citation: Podolskaya, M.S., Tkachuk, A.A., Andreyeva, A.Yu., Kladchenko, E.S., Chelebieva, E.S. and Mosunov, A.A., 2023. Effect of Bicomponent $ZnO-ZnFe_2O_4$ Nanoparticles on Mediterranean Mussel (*Mytilus galloprovincialis*) Hemocytes under *in vitro* Conditions. *Ecological Safety of Coastal and Shelf Zones of Sea*, (1), pp. 124–136. doi:10.29039/2413-5577-2023-124-136

Влияние бикомпонентных наночастиц $ZnO-ZnFe_2O_4$ на гемоциты средиземноморской мидии (*Mytilus galloprovincialis*) в условиях эксперимента *in vitro*

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Аннотация
Исследовано токсическое действие бикомпонентных наночастиц $ZnO-ZnFe_2O_4$, являющихся основным действующим компонентом отечественного противообрастающего покрытия, на маркерные показатели клеток гемолимфы (гемоциты) средиземноморской мидии (*Mytilus galloprovincialis*) в экспериментальных условиях *in vitro*. Оценивались следующие показатели: смертность, клеточный состав и продукция активных форм кислорода. В эксперименте гемоциты инкубировали в течение 1 и 2 ч в 1 мл стерильной морской воды, содержащей наночастицы в различных концентрациях: 0.03, 0.3 и 3 мг/мл. Данные анализировали с помощью метода проточной цитометрии. Установлено, что действие наночастиц $ZnO-ZnFe_2O_4$ оказало влияние на клеточный состав гемолимфы: снижалась доля агранулоцитов, воздействие 0.03 мг/мл наночастиц при часовой инкубации снижало уровень продукции активных форм кислорода в 2.5 раза по сравнению с контрольной пробой ($p \leq 0.05$). Инкубация гемоцитов с максимальной концентрацией наночастиц (3 мг/мл) привела к гибели клеток уже через 1 ч после воздействия. Острого токсического воздействия на гемоциты при применении 0.03 мг/мл и 0.3 мг/мл наночастиц оксида цинка и феррита цинка не наблюдалось.

Ключевые слова: наночастицы, средиземноморская мидия, гемоциты, АФК

Благодарности: синтез наночастиц ($ZnO-ZnFe_2O_4$) был выполнен в рамках выполнения проекта РНФ № 21-13-00498 «Экологически безопасные и высокоэффективные противообрастающие покрытия на основе бикомпонентных наночастиц металлов и их оксидов». Оценка токсичности наночастиц на организм мидий (анализ параметров гемоцитов) проводился за счет средств госзадания ФИЦ ИнБЮМ № 121102500161-4 «Закономерности организации иммунной системы промысловых гидробионтов и исследование влияния факторов внешней среды на функционирование их защитных систем».

Для цитирования: Влияние бикомпонентных наночастиц $ZnO-ZnFe_2O_4$ на гемоциты средиземноморской мидии (*Mytilus galloprovincialis*) в условиях эксперимента *in vitro* / М. С. Подольская [и др.] // Экологическая безопасность прибрежной и шельфовой зон моря. 2023. № 1. С. 124–136. EDN BPTQRT. doi:10.29039/2413-5577- 2023-124-136

Introduction

Biofouling is a natural process involving the attachment of micro- and macroorganisms to various substrates in the aquatic environment [1)]. Moreover, habitation of fouling organisms on the surfaces of hydraulic structures causes significant damage to shipping, oil platforms and industry as a whole [1, 2]. Global damage from marine fouling currently exceeds $50 billion per year [3]. The main task of antifouling coatings is to prevent the formation of a biological film, which is the first stage of biofouling [4]. At the same time, such coatings must be safe with respect to non-target organisms. Antifouling coatings widely used in the past were based on such biocides as tributyltin and copper, which proved to be highly toxic to marine organisms and were disallowed [5–7].

One of the innovative methods of protection against biofouling is the use of coatings based on metal oxide nanostructures [8]. The most commonly used nanoparticles are of such metals as zinc, aluminum, iron, titanium, silver and copper, as well as their oxides [9]. These nanoparticles have proven antibacterial, anticorrosive and antifouling properties [10].

Their low toxicity to aquatic organisms is an important quality of new antifouling components. Nevertheless, the data available in the publications are insufficient to make an unambiguous conclusion about the toxicity of nanoparticles to marine organisms. It is known that the toxicity of nanoparticles depends on the type of metal, size, shape and surface charge of the particles [11]. Among possible mechanisms of the toxic effect of nanoparticles on the organisms of hydrobionts, it is possible to single out the production of reactive oxygen species (ROS), which is a consequence of the disproportionately large surface area of nanoparticles [12]. ROS overproduction, which exceeds the ability of cells to provide antioxidant protection, causes oxidative stress, which leads to disturbances in the structure of the cell membrane, mitochondria, and oxidation of proteins and DNA [13, 14]. Thus, a study of aluminum oxyhydroxide (boehmite) exposure to nanoparticles revealed no acute toxicity to *Daphnia magna* (Straus, 1820) [15]. At the same time, the study of titanium dioxide nanoparticles showed the ability of nanoparticles to accumulate in the bodies of daphnia [16]. The impact of copper oxide nanoparticles led to the behavior disorder of the mollusk *Scrobicularia plana* (da Costa, 1778) [17]. Also, the impact of zinc oxide nanoparticles had a negative influence on the development of marine fish *Oryzias melastigma* larvae (McClelland, 1839) [18].

Currently, the development of antifouling coatings based on nanoparticles is considered to be a promising area. Previously, a new antifouling paint component was created, which contained ZnO-$ZnFe_2O_4$ nanoparticles obtained

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[1)] Korobkov, V.A., Levin, V.S., Lukoshkov, A.V. and Serebrenitsky, P.P., 1981. [*Underground Technology*]. Leningrad: Sudostroenie, 240 p. (in Russian).

by electrical explosion of twisted zinc and iron wires in oxygen-containing atmosphere. ZnO nanoparticles are able to absorb visible light and inhibit the growth of microorganisms due to the photocatalytic process [19]. The composition is a domestic development and has proven antifouling properties [20], but there is no data on its safety in relation to marine organisms. Since the method we use to obtain nanoparticles is quite original [21], and, as we know, the particles of the studied composition were obtained for the first time, detailed studies of their physical and chemical properties, as well as the mechanisms of biological action, provide the subject for our future research.

Bivalve molluscs are convenient model objects to study the effect of nanoparticles, as they lead an attached mode of life and are filter-feeding organisms [22]. The functional state of molluscs is assessed by morphological and physiological parameters of hemolymph cells, hemocytes, which, in turn, are cellular immunity effectors [23]. The cellular immune response is based on the production of reactive oxygen species (ROS) by hemocytes, phagocytosis and encapsulation of foreign substances [24, 25]. Mollusc hemocytes react to the action of stimuli with a respiratory burst, which represents a sharp increase in the production of ROS by cells that cause oxidative stress in pathogenic organisms [26]. Hemocytes are a generally accepted model to assess the physiological state of molluscs [6].

Studying the toxicity of new paint components based on nanoparticles, determining acceptable concentration ranges, and understanding the effect of nanoparticles on immune system effectors form a relevant objective. In connection with the above, the purpose of this work is to evaluate the toxic effect of ZnO-$ZnFe_2O_4$ nanoparticles on the functional parameters of hemocytes of the hemolymph of the bivalve mollusc *Mytilus galloprovincialis*.

Materials and Methods

Mature, four years old mussels (*M. galloprovincialis*) weighing 12.9 ± 2.3 g, 57.8 ± 1.8 mm in size, were selected in the amount of 210 pieces in the Sevastopol coastal area (water temperature 15–20 °C, salinity 17–18 PSU, oxygen content 7.2–8.5 mg/L). The mussels were delivered to the laboratory in plastic containers with no water. The molluscs were acclimated to laboratory conditions in aquaria with a stocking density of 3–5 L per individual for 7 days. The conditions close to the point of material collection were maintained in the aquaria: temperature 23.3 ± 0.1 °C; salinity 18.2 ± 0.02 PSU; pH 8.1 ± 0.01; oxygen ontent 7.7 ± 0.1 mg/L. The oxygen content and water temperature were monitored using a portable oxygen meter with a temperature sensor *ST*300*D* (Ohaus, USA). Salinity and pH were controlled using a portable conductometer-salinometer sensION 5 HACH (USA) and pH-meter ST2100-F (Ohaus, USA). Throughout the experiment, including the period of acclimation to laboratory conditions, the water was changed daily to remove metabolites while maintaining the salinity value. The molluscs were fed with a mixture of microalgae *Tetraselmis viridis* (strain IBSS-25 from the collection of the IBSS FRC Department of Biotechnology and Phytoresources)

at a rate of 5–10 mL of the mixture for every 50 L of aquarium water. After acclimation, the hydrobionts were divided into 7 groups of 30 individuals in each group (one control group, three groups with incubation for 1 hour (groups 1.1, 1.2, and 1.3), three groups with incubation for 2 hours (groups 2.1, 2.2, and 2.3).

The effect of nanoparticles on mollusk hemocytes was studied under *in vitro* conditions. For this purpose, hemolymph was taken with a sterile syringe from the adductor muscle of 10 individuals and combined into one sample. To obtain hemocytes, the composite sample was washed three times in sterile sea water by centrifugation (500 g, 5 min). The pellet was used for further analysis. All work on washing and preparing cells for analysis was carried out at +4 °C to prevent cell adhesion.

In the experiment, the cells were incubated in 1 mL of sterile sea water containing nanoparticles. Each sample contained $1 \cdot 10^6$ cells/mL. Different concentrations of bimetallic ZnO-$ZnFe_2O_4$ nanoparticles synthesized under the Russian Science Foundation project were used: 0.03 (the concentration is 10 times lower than the active one), 0.3 (the active concentration of the developed antifouling mixture) and 3 (the concentration is 10 times higher than the active one) mg/mL. Cells were incubated in a thermoshaker with cooling for microtubes (Biosan TS-100C) at +4 °C and 1,000 rpm. One part of the hemocyte samples in the range of the studied concentrations of nanoparticles (groups 1.1 (the concentration of nanoparticles in the sample is 0.03 mg/mL), 1.2 (0.3 mg/mL), and 1.3 (0.03 mg/mL)) was incubated for 1 h, the other one (groups 2.1 (the concentration of nanoparticles in the sample is 0.03 mg/mL), 2.2 (0.3 mg/mL), and 2.3 (0.03 mg/mL)) – for 2 h. A sample of hemocytes in sea water without nanoparticles served as a control. After incubation, the cells of the experimental samples were washed from nanoparticles in sterile sea water by centrifugation (500 g, 5 min).

Analysis of the functional parameters of hemocytes was carried out on a flow cytometer Cytomics FC 500 (Beckman Coulter, USA), equipped with a single-phase argon laser (wavelength 488 nm). For analysis by flowing cytometry, a suspension was prepared with a concentration of hemocytes $(1–2) \cdot 10^6$ cells per 1 mL.

To identify the types of hemocytes, the prepared suspension of hemolymph cells was stained with the dye SYBR Green I (SGI). The final concentration of SGI in the sample makes 10 μmol/L. Stained cells were incubated for 40 min in the dark at +4 °C. The content of DNA in hemocytes was analyzed on the basis of histograms of the dye fluorescence distribution in channel *FL*1 using the program *Flowing Software* 5.2. The abscissa axis on the histogram of the dye fluorescence distribution displayed the DNA content in the cells, and the ordinate axis – the number of cells.

The ability of hemocytes to spontaneous production of ROS was evaluated based on the assessment of the fluorescence intensity of the 2-7-dichlorofluorescein-diacetate (*DCF-DA*) dye. Then, 1 mL of hemocyte suspensions were incubated with 10 μl of *DCF-DA* solution for 40 min in the dark. The final dye concentration in the sample made 10 μmol/L. The dye fluorescence was analyzed in channel *FL*1.

Hemocyte survivorship rate was determined using propidium iodide (PI), a fluorescent dye for nucleic acids. 10 µl of PI solution (Sigma Aldrich) was added to 1 mL of hemocyte suspension and incubated in the dark for 40 min at 4 °C. The proportion of dead cells in the total number of hemocytes was estimated from PI fluorescence histograms in channel *FL2* of the cytometer.

When processing the results, the normality of the distribution was controlled using the Kolmogorov–Smirnov test. Differences among the groups were analyzed using *RStudio* software version 4.1.0.

The distribution of functional parameters of hemocytes did not obey the normal distribution law, so the data were analyzed using the nonparametric Mann–Whitney test. The results are expressed as mean ± standard error of the mean.

Results

Significant differences between the control (hemocyte sample in sea water without nanoparticles) and experimental (hemocyte samples with different concentrations of nanoparticles and incubation time (groups 1.1, 1.2, 1.3, 2.1, 2.2 and 2.3) groups of hemocytes in terms of the number of dead cells were not detected, although there was a tendency towards an increase in their number as a result of exposure to nanoparticles (Fig. 1).

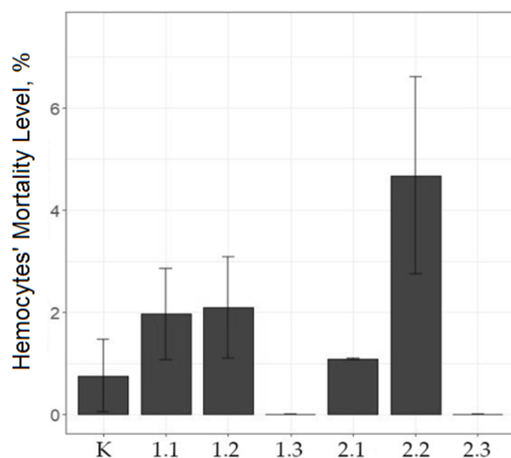
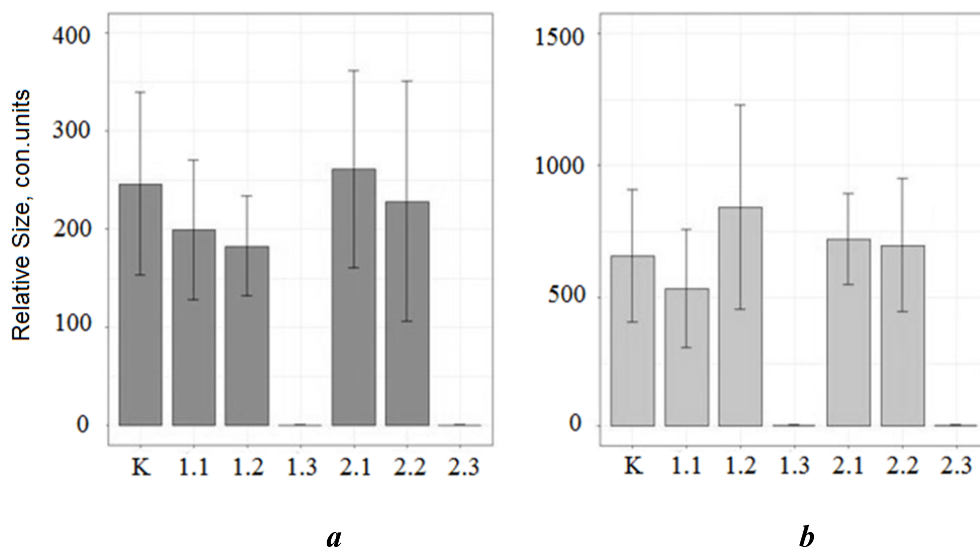


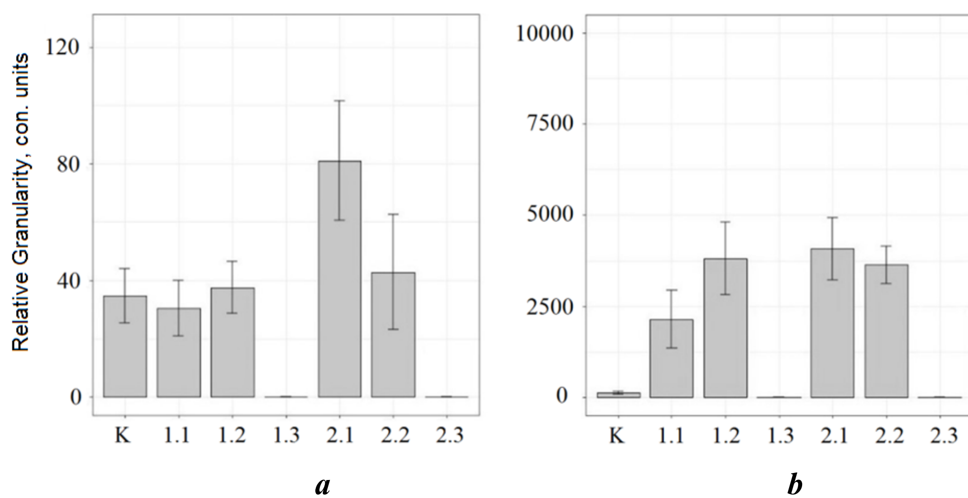
Fig. 1. Effect of ZnO-$ZnFe_2O_4$ nanoparticles on the mortality of Mediterranean mussel (*M. galloprovincialis*) hemocytes. K – control; 1.1 – incubation 1 h, concentration 0.03 mg/mL; 1.2 – incubation 1 h, concentration 0.3 mg/mL; 1.3 – incubation 1 h, concentration 3 mg/mL; 2.1 – incubation 2 h, concentration 0.03 mg/mL; 2.2 – incubation 2 h, concentration 0.3 mg /mL; 2.3 – incubation 2 h, concentration 3 mg/mL

Since the dye enters the cells through the damaged membrane, the absence of data on the diagram at the maximum concentration (3 mg/mL) indicates that the hemolymph cells were destroyed and died right after the first hour of exposure and were removed by other hemocytes through phagocytosis.

Fig. 2 and 3 show the effect of nanoparticles on the size and granularity of mussel hemolymph cells. No significant differences revealed among the groups may indicate that the nanoparticles present in the samples did not have any significant effect on the size and granularity of hemocytes. However, there was a tendency towards a decrease in the size of agranulocytes with an increase in the concentration of nanoparticles.



F i g . 2 . Effect of $ZnO\text{-}ZnFe_2O_4$ nanoparticles on the relative size of hemocytes of Mediterranean mussel (*M. galloprovincialis*): *a* – that of agranulocytes; *b* – that of granulocytes. For the other nomenclatures see Fig. 1



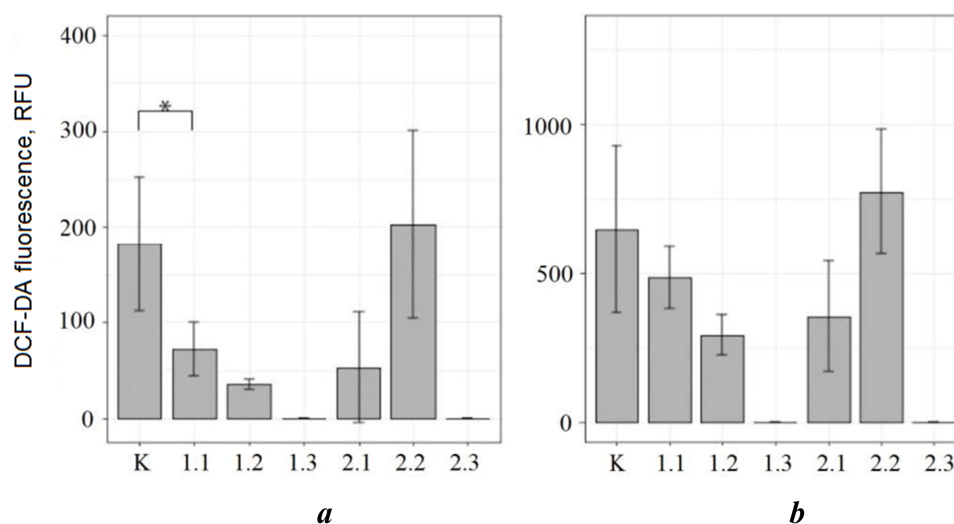
F i g . 3 . The effect of $ZnO\text{-}ZnFe_2O_4$ nanoparticles on the relative granularity of Mediterranean mussel (*M. galloprovincialis*) hemocytes: *a* – that of agranulocytes; *b* – that of granulocytes. For the other nomenclatures see Fig. 1

As a result of the incubation of hemocytes with nanoparticles, there were slight changes in the level of relative granularity of hemocytes: the values of lateral scattering in agranulocytes increased with an increase in the concentration of nanoparticles, while in granulocytes, on the contrary, there was a tendency to an increase in the level of the cytoplasm granularity depending on the concentration (Fig. 3). At the same time, changes in the values of side scattering between the control and experimental groups were not significant.

Nanoparticles affected the spontaneous production of ROS by hemolymph cells significantly. The fluorescence intensity of agranulocytes and granulocytes stained with *DCF-DA* fluorescent dye decreased depending on the concentration of nanoparticles in the medium (Fig. 4). The production of ROS by agranulocytes decreased by 2.5 times from 182.3 ± 69.7 relative fluorescence units (RFU) in the control sample to 72.1 ± 28.2 RFU in a sample with an hourly incubation containing nanoparticles with the concentration of 0.03 mg/mL ($p \leq 0.05$). An increase in the production of ROS was observed only during a two-hour *incubation* when both populations of cells were exposed to nanoparticles with the concentration of 0.3 mg/mL. Nevertheless, the changes were statistically insignificant.

Discussion

The results of the work indicate that the active concentration of nanoparticles (0.3 mg/mL) did not have any significant effect on the hemocytes of the Mediterranean mussel *M. galloprovincialis*. No significant differences in the functional parameters of cells were revealed between the control and experimental groups.



F i g . 4 . The effect of $ZnO\text{-}ZnFe_2O_4$ nanoparticles on DCF-DA fluorescence intensity of the Mediterranean mussel (*M. galloprovincialis*) hemocytes: *a* – in agranulocytes, relative fluorescent units, RFU; *b* – in granulocytes, RFU. For the other nomenclatures see Fig. 1

There was a tendency towards an increase in the proportion of granulocytes and a decrease in the proportion of agranulocytes. A slight increase in cell size and granularity was also observed. The concentration of nanoparticles 10 times lower than the active one (0.03 mg/mL) also had no significant effect on the functional parameters of mussel hemocytes. At the same time, exposure to nanoparticles during one-hour incubation reduced the level of the production of ROS by 2.5 times compared to the control sample. Incubation of hemocytes with a concentration of nanoparticles 10 times higher than the active one (3 mg/mL) led to cell death as early as 1 h after exposure.

It is known that hemocytes of bivalve molluscs play an important functional role in the organism, being responsible for cell-mediated immunity through phagocytosis and production of cytotoxic molecules of various nature [23]. During the incubation of hemocytes with nanoparticles, there was a tendency to increase the size and degree of granularity of hemocytes, which may indicate active phagocytosis of nanoparticles. In this case, the formation of phagosomes with nanoparticles led to an increase in the heterogeneity of the content of the hemocyte cytoplasm and, as a consequence, to an increase in the lateral scattering index. Changes in the shape of cells containing different amounts of nanoparticles also affected the amount of sample direct scattering. Intense absorption of nanoparticles by hemocytes is likely to be able to reduce the overall phagocytic capacity of hemocytes, which can lead to the weakening of the innate immune system of bivalves [27]. Similarly to our results, the effect of Ag and TiO_2 nanoparticles on the hemolymph of the oyster *Crassostrea virginica* also led to a decrease in cell phagocytosis after 2 h of exposure [28].

An increase in the concentration of nanoparticles in the one-hour incubation group led to a decrease in the production of ROS, while in the two-hour group, on the contrary, it induced the production of ROS (Fig. 4). Since nanoparticles are able to independently generate ROS, an increase in ROS content in cells should be considered as the development of oxidative stress in hemocytes [29, 30]. It is also known that nanoparticles taken up by hemocytes can affect immune functions such as phagocytosis and production of ROS [31]. At the same time, it was revealed that phagocytosis of foreign particles causes an increase in the production of ROS [32]. Mitochondria are the main source of ROS [33]. The production of ROS by hemocytes is an important protective function of the innate immunity of bivalves. Once inside the cell, nanoparticles can depolarize the mitochondrial membrane and disrupt its functions [29]. In response to this exposure, mitochondria increase the production of ROS, causing oxidative stress [34]. As it is known, phagocytosis is an energy-consuming process, and this can also be the cause of ROS generation [35]. Previously, a study of the effect of carbon soot nanoparticles on hemocytes of the mussel *M. galloprovincialis* under *in vitro* conditions for 0.5–4 h (1–10 μg/mL) also showed an increase in the production of ROS in the cell [23]. On the contrary, in another study by the same researcher devoted to the effect of TiO_2 and SiO_2 nanoparticles on the mussel hemocytes, no significant toxic effect on the cell was revealed [36]. When mussel hemocytes were exposed to TiO_2 nanoparticles, a significant increase in the production of ROS

and a decrease in the membrane potential of mitochondria, which were observed when exposed to maximum concentrations, were noted [35].

Conclusion

Thus, it can be concluded that the toxicity of nanoparticles mainly depended on their concentration and time of exposure. The results showed that an acute toxic effect occurred with a concentration of 3 mg/mL, at which cell death took place within an hour of incubation. In groups 1.1, 1.2 and 1.3 with hourly incubation, a decrease in the production of ROS was observed, and in groups 2.1, 2.2 and 2.3 with two-hour incubation, on the contrary, it increased, which may indicate the mechanisms of cell adaptation to stress. No specific cell response was observed when exposed to nanoparticles with concentrations of 0.03 and 0.3 mg/mL, which makes it possible to use such concentrations in the aquatic environment.

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Submitted 9.08.2022; accepted after review 5.12.2022;
revised 1.02.2023; published 24.03.2023

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