NANOBIOLOGY
AND GENETICS, OMICS

The Survival of the Infusoria *Paramecium caudatum* in the Presence of Aluminum Oxide Nanoparticles

M. A. Kryuchkova[a,*]**, F. S. Akhatova**[a]**, and R. F. Fakhrullin**[a]

[a]*Kazan (Volga Region) Federal University, Kazan, Russia*
***e-mail: maricshka80@gmail.com**

Abstract—Acute toxicity of nanoparticles of aluminum oxide (Al_2O_3) with a size of 13−16 nm was investigated by the biotesting method using *Paramecium caudatum* ciliates in the concentration range of 10−100 μg/mL. Aluminum oxide has an acute toxic effect on paramecium at concentrations of 20−100 μg/mL. The mean lethal dose (LD_{50}) is equal to the concentration of nanoparticles at which the mortality of ciliates in relation to the control reached 50%. The LD_{50} for Al_2O_3 nanoparticles is 23 μg/mL at a 24-h exposure. According to published data, the toxic effect of Al_2O_3 nanoparticles is specific and depends on the size and surface charge of the particles and on the interfacial interaction of nanoparticles with the cell surface, as well as on the concentration and exposure time.

INTRODUCTION

The exceptional properties of nanomaterials determine the prospects for their wide application in industry [1]. Metal oxide nanoparticles (NPs) are widely used in the production of structural materials, catalysts, energy storage devices, paints, phosphors, cosmetic, and medical preparations [2, 3]. One of the important directions in the development of nanotechnology is the production of nanopowders, 80% of which are powders of metal oxides; the most popular and in demand for production are NPs of aluminum oxide (Al_2O_3) [4]. Aluminum oxide is used for the manufacture of optically transparent and structural ceramics, heat-protective coatings and paints and varnishes, acts as a catalyst in a number of organic synthesis processes [5, 6]. Due to the large production of Al_2O_3 and the open nature of many technological cycles in which it is used, this nanomaterial can be a significant pollutant and pose a serious threat to the environment [7]. It should be noted that the toxic effect from particles of the nanorange is much greater than from particles of similar composition of micron sizes [8, 9].

Despite its prevalence, aluminum and its compounds are toxic elements [10]. Al_2O_3 NPs are easily absorbed by various cell cultures [11, 12] while exerting a cytotoxic effect [12, 13] and possess the ability to catalytically generate free radicals [14]. Alumina dust (~33 g/m³ five hs a day) causes severe damage to the epithelium of the respiratory tract of rats [15].

Aluminum and its compounds enter natural waters during the partial dissolution of clays and aluminosilicates, as well as a result of harmful emissions from industrial enterprises and with wastewater [15]. Every year there are more such emissions into the environment; control over the degree of pollution by them is getting lower. Since Al_2O_3 NPs are insoluble in water and virtually incapable of biological degradation, they can accumulate in the components of natural ecosystems and have a detrimental effect on most living organisms that inhabit natural water bodies [16]. The harmful effect of aqueous dispersions of Al_2O_3 on daphnia [17], freshwater snails [18], fish [19], soil nematodes [20], and insects [21] has been shown.

The main methods for monitoring the ecological state of water bodies continue to be physicochemical methods. However, along with analytical methods, biological testing methods are increasingly being used, which make it possible to assess the entire set of properties of the studied environment by the responses of living organisms. Freshwater ciliates are such organisms; they are widespread in water bodies and play a significant role in the self-purification of water. Being unicellular organisms, ciliates simultaneously demonstrate reactions at the organism and cellular levels, thereby expanding the range of criteria for assessing toxicity. Unfortunately, the issues of the reaction of ciliates to Al_2O_3 has been insufficiently studied.

The main goal of this study is to determine the toxic effect of aluminum oxide nanoparticles in an experiment on the infusoria *Paramecium caudatum* (*P. caudatum*).

EXPERIMENTAL

To assess the toxicity of Al_2O_3 NPs, we used a commercial preparation from Sigma-Aldrich, which is a white nanodispersed powder with a particle diameter of 16.4 ± 10.0 nm and a ζ potential of 44.3 ± 1.8. The ζ potential and hydrodynamic particle diameter were measured using a Zetasizer Nano ZS instrument (Malvern).

Al_2O_3 NPs were characterized using dark-field electron microscopy (TEM) and atomic force microscopy (AFM). AFM images were obtained with a Dimension Icon microscope (Bruker) operating in PeakForce Tapping mode, using a ScanAsyst-Air probe (Bruker) (nominal length 115 μm, tip radius 2 nm, spring rate 0.4 N m^{-1}). The obtained data were processed using the Nanoscope Analysis v.1.7 software. (Bruker). Al_2O_3 NPs were visualized using high-contrast TEM CytoViva®. TEM images were obtained with a condenser CytoViva®attached to an Olympus BX51 microscope equipped with a fluorite objective (×100) and CCD-camera.

For biotesting, an aqueous suspension of Al NPs$_2O_3$ was prepared just before the study. To eliminate aggregation, the suspension was sonicated (for 2 min at 44 kHz and 40 W). The acute toxicity of Al_2O_3 NPs was investigated at concentrations of 100, 50, 40, 30, 25, 20, 15, and 10 μg/mL at various exposures (0.16, 0.5, 1, 3, 5, and 24 h).

We used equalciliary ciliates *P. caudatum* as model organisms. Assessment of the resistance of ciliates to Al_2O_3 NPs was carried out according to the method [22] based on determining the survival rate of ciliates. *P. caudatum* were cultivated in a ten-fold dilution of the Lozin-Lozinsky medium prepared by dissolving the following weighed portions of salts in 1 L of distilled water: NaCl (1.0 g), KCl (0.1 g), NaHCO$_3$ (0.2 g), MgSO$_{four}$ (0.1 g), and CaCl$_2$ (0.1 g) with the addition of an aqueous yeast suspension of *Saccharomyces cerevisiae* (3 ml) at a temperature of $22-24°C$. For the experiments, ciliates were selected manually using a micropipette. A Carl Zeiss Stemi 2000C stereoscopic microscope was used to observe the paramecium. A culture plate with wells was used for biotesting. Using a micropipette, $10-12$ individuals were selected in a minimal amount of medium and transferred to the wells of the plate. After placing the ciliates in the plates, 0.2 ml of the culture medium was poured into the control wells and 0.2 ml of the test sample were added to the experimental wells. The start time of the biotesting was noted. During the exposure, the ciliates were not fed either in the control or in the experimental wells. The criterion of toxicity was the death of ciliates. Immobile and reshaped cells were considered dead. In addition, change in the nature of movement of ciliates was assessed.

Survival of the ciliates (N, %) was determined by the formula

$$N = N_2/N_1 \times 100,$$

where N_2, N_1 is the arithmetic mean of the number of ciliates at the end and beginning of the experiment, pcs.

The average lethal dose (LD$_{50}$) is defined graphically as the concentration of the test solution at which the toxicity is 50%.

All experiments were performed in triplicate. Moreover, each series of experiments was performed at least three times. We used the *t*-Student's test for the statistical processing of the results. Differences were considered significant for $p \leq 0.05$.

RESULTS AND DISCUSSION

This study aimed to investigate the potentially toxic effects caused by Al_2O_3 NPs, which are now widely used in industry. Typical AFM images show the geometry and size of Al_2O_3 particles, which are ellipsoidal and $13-16$ nm in diameter. These images represent the topography of the surface as much as possible. For visualization of Al_2O_3 particles, TEM was used (Fig. 1).

To assess the toxicity of Al_2O_3 *P. caudatum*, which are motile unicellular microscopic organisms that feed on yeast and capture other particles suspended in an aqueous medium, were used as a model in vivo. Paramecia have a typical ellipsoid shape, the cells themselves are transparent, which makes it possible to visualize organelles, for example, digestive vacuoles filled with yeast cells (Fig. 2a). Enhanced TEM can be used to observe the absorption of Al_2O_3 particles. From Fig. 2b it can be seen that particles of Al_2O_3 fall into the cell of *P. caudatum* from the aquatic environment. Aluminum oxide inhibits phagocytic activity in *P. caudatum*, in this case, the process of formation of digestive vacuoles is disrupted in the entire range of the studied concentrations. At lower concentrations of Al_2O_3 NPs were diffusely distributed in the cytoplasm (Fig. 2b). As an example, silicon oxide at a concentration of 5 mg/mL has a slightly toxic effect [23], but it does not prevent the formation of digestive vacuoles even at high concentrations. After entry, silicon oxide NPs are transferred to the digestive vacuoles and visualized by TEM (Fig. 2c).

High-contrast TEM images allow rapid, simple, and efficient observation of Al_2O_3 NPs inside the transparent bodies of paramecium, as in previous studies with the microscopic worms *Caenorhabditis elegans* [24] and ciliates *P. caudatum* [23].

In a typical experiment, the acute toxicity of Al_2O_3 NPs was assessed in the concentration range from 10 to 100 μg/mL. At the same time, the survival rate of ciliates was investigated at various exposures (Table 1).
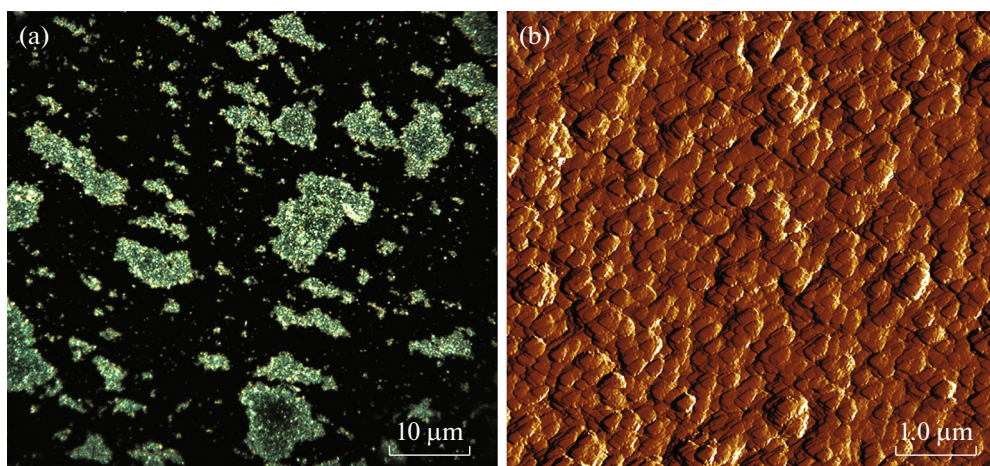
Fig. 1. (Color online) Aluminum oxide nanoparticles: (a) dark field microscopy; (b) atomic force microscopy.
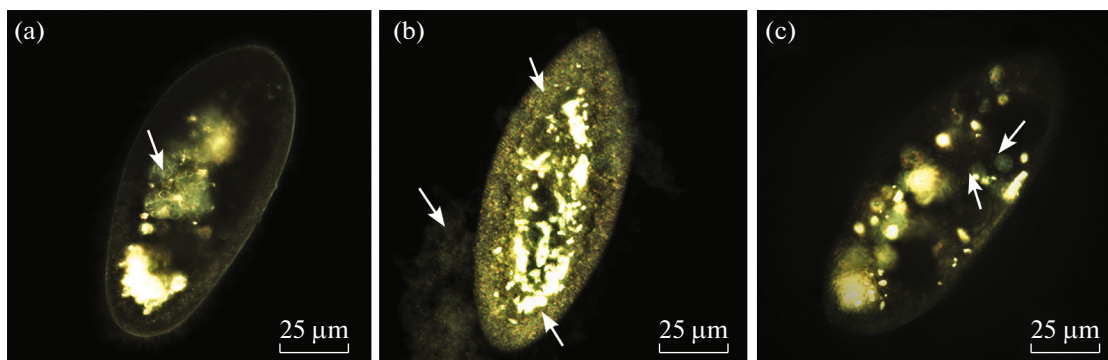

Fig. 2. (Color online) TEM images of ciliates *P. caudatum*: (a) control (the arrow indicates the digestive vacuoles filled with yeast cells), (b) absorption of nanoparticles of aluminum oxide *P. caudatum* (indicated by an arrow), NP distribution visualization of Al_2O_3 in the cytoplasm of *P. caudatum* (indicated by arrows); (c) visualization of the digestive vacuoles filled with silica particles (indicated by arrows).

To assess the survival rate, the number of dead individuals was taken into account. Deformation of the body, rupture of the membrane, lysis of the cell, as well as preservation of immobility, are indicators of the death of ciliates. After 0.16 h of the experiment at concentrations of 100 and 50 µg/mL the absence of motor activity in 50% of the cells was noted. After 0.5 h at a concentration of 100 µg/mL, rupture of the cell membrane and cell lysis were observed, while at a concentration of 50 µg/mL, approximately 30% of the cells retained locomotor activity. In other concentrations, most of the cells retained normal locomotor

Table 1. The survival of ciliates *P. caudatum* (%) depending on the concentration of aluminum oxide

C, µg/mL	0.16 h	0.5 h	1 h	3 h	5 h	24 h
100	34.2 ± 3.2	0	0	0	0	0
50	52.2 ± 3.6	29.2 ± 4.4	29.2 ± 4.2	21.9 ± 4.9	14.6 ± 4.9	14.6 ± 4.8
40	64.3 ± 3.4	42.9 ± 4.6	35.7 ± 4.3	28.6 ± 4.3	28.6 ± 4.2	21.4 ± 3.5
30	81.8 ± 4.4	63.6 ± 4.5	45.7 ± 4.5	45.4 ± 4.8	45.4 ± 4.3	42.4 ± 4.5
25	81.4 ± 3.8	70.8 ± 3.6	61.9 ± 3.6	53.0 ± 4.2	52.3 ± 4.7	44.2 ± 4.7
20	81.8 ± 3.7	81.8 ± 4.1	72.7 ± 3.9	64.5 ± 2.8	63.0 ± 2.9	60.5 ± 3.2
15	90.4 ± 2.3	86.7 ± 2.6	86.2 ± 2.8	78.3 ± 2.5	76.9 ± 2.9	75.9 ± 2.9
10	95.2 ± 2.0	92.6 ± 2.2	90.4 ± 2.3	88.3 ± 2.7	87.2 ± 2.7	85.6 ± 2.5
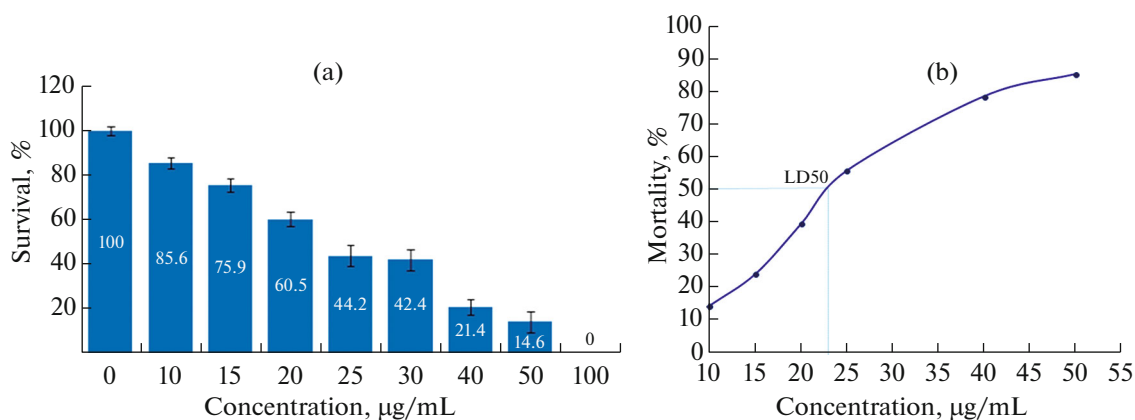
Fig. 3. (Color online) The toxicity of aluminum oxide on ciliates *P. caudatum*: (a) survival of ciliates after 24 h of incubation with aluminum oxide, (b) LD_{50} the lethal dose of aluminum oxide that causes the death of 50% of the ciliates in 24 h.

activity. The survival rates of ciliates after 24 h of incubation with Al_2O_3 are presented in Fig. 3a. The greatest decrease in survival was observed at concentrations of 20−100 μg/mL, at which Al_2O_3 has an acute toxic effect on ciliates. Concentrations of 10 and 15 μg/mL have a slightly toxic effect on Paramecium.

The LD_{50} is the lethal dose of Al_2O_3 that causes the death of half (50%) of the organisms within a certain period of time (24 h). As a result of the studies, the toxicity parameter of Al_2O_3 under acute exposure was $LD_{50} = 23$ μg/mL (Fig. 3b).

In studies by other authors, Al_2O_3 NPs were less toxic. It was found in [25] that the concentration of Al_2O_3 (particle size 83 nm), at which 50% mortality occurs in the ciliates *Paramecium multimicronucleatum* in a 48-h exposure was 9269 mg/L. The toxicity of NP Al_2O_3 (with a particle size Δ_{50} of 7 and 70 nm) was studied by the chemotoxic response of ciliates *P. caudatum* [26]. According to the conducted studies Al_2O_3 NPs ($\Delta_{50} = 70$ nm) turned out to be more toxic ($LD_{50} = 1.22$ mg/L) than ($\Delta_{50} = 7$ nm) NPs, which had no toxic effect.

Inconsistency of the data on the dependence of the toxicity of Al_2O_3 NPs on particle size can be caused by the authors' use of various toxicity assessment methods.

The toxicity of Al_2O_3 depends on the interfacial interaction of NPs with the cell surface, as well as on the physicochemical properties of NPs (size and surface charge) [25]. With a decrease in NP size, the surface area increases, which causes a dose-dependent increase in oxidative stress [27]. Oxidative stress is one of the main mechanisms of the toxic effect of Al_2O_3 NPs under the influence of NPs on aquatic organisms [26]. Particle charge is also a significant factor. Positively charged particles with high affinity for DNA macromolecules which, therefore, carry a genotoxic potential, are the most dangerous [27].

The toxicity of Al_2O_3 NPs is different for different test organisms. The inhibitory effect of Al_2O_3 with a particle size of 70 nm for the growth of microalgae *Chlorella vulgaris* ($LD_{50} = 15$ mg/L), and for *Daphnia magna* Al_2O_3 turned out to be less; the LD_{50} is more than 100 mg/L [26]. Aluminum oxide (with a particle size of 16 nm) at a concentration of 4 mg/L induced irreversible histopathological lesions of the branchial, liver, and brain tissues of freshwater fish *Oreochromis mossambicus* after 96 h of exposure [19]. When comparing the median lethal dose of Al_2O_3 in different species it was found that *P. caudatum* ciliates are more sensitive organisms for assessing the toxicity of Al_2O_3 in aqueous media ($LD_{50} = 23$ μg/mL).

When analyzing the literature data, it can be concluded that the toxic effect of Al_2O_3 NPs is specific and depends on the size and surface charge of the particles, as well as on the concentration and exposure time.

Thus, the high toxicity of Al_2O_3 NPs can be caused by the small size of particles (13−16 nm) and their high penetrating ability, which facilitates their redistribution within the cell. The aluminium oxide, used in this study has a positive surface potential (44 mV), which can also contribute to increased toxicity. Al_2O_3 NPs are capable of generating reactive oxygen species, damaging DNA, disrupting protein expression, depolarizing the cell membrane, and causing morphological changes and cell death [12, 28]. Aluminum oxide has a harmful effect on lower aquatic organisms involved in the self-purification of water bodies that are food resources for fish. Consequently, the contamination of the aquatic environment with Al_2O_3 NPs can have a negative impact on living organisms and pose a threat to aquatic ecosystems.

Despite the fact that ciliates are widely used as a model object to assess toxicity, the mechanisms of toxic effects of Al_2O_3 NPs on the *P. caudatum* have

practically not been studied. This indicates the need for additional toxicological studies.

CONCLUSIONS

The danger of nanoparticles of aluminum oxide was assessed by the survival rate of ciliates *P. caudatum*. Al_2O_3 NPs have an acute toxic effect on paramecium. At a concentration of 100 μg/mL (exposure 0.5 h), 100% death of ciliates is observed. The LD_{50} is 23 μg/mL at a 24-h exposure. The relatively high toxicity Al_2O_3 NPs can be caused by the small size (13–16 nm) and positive charge of the particles, as well as their high penetrating ability, which facilitates their redistribution within the cell.

FUNDING

REFERENCES

1. R. Chatterjee, Environ. Sci. Technol. **2**, 339 (2008). https://doi.org/10.1021/eS0870909

2. J. Gangwar, B. K. Gupta, and A. K. Srivastava, Defense Sci. J. **66**, 323 (2016). https://doi.org/10.14429/dsj.66.10206

3. A. Yu. Godymchuk, G. G. Savel'ev, and A. P. Zykova, *Ecology of Nanomaterials, The School-Book* (BINOM, Labor. Znanii, Moscow, 2012) [in Russian].

4. O. A. Zeinalov, S. P. Kombarova, D. V. Bagrov, et al., Obz. Klin. Farmakol. Lek. Ter. **14** (3), 24 (2016). https://doi.org/10.17816/RCF14324-33

5. N. Roussel, L. Lallemant, J. Y. Chane-Ching, et al., J. Am. Ceram. Soc. **96**, 1039 (2013). https://doi.org/10.1111/jace.12255

6. V. V. Ivanov, A. S. Kaigorodov, V. R. Khrustov, et al., Ross. Nanotekhnol. **1** (1–2), 201 (2006).

7. A. A. Shumakova, O. N. Tananova, E. A. Arianova, et al., Vopr. Pitan. **81** (6), 54 (2012).

8. H. Ma, P. L. Williams, and S. A. Diamond, Environ. Pollut. **172**, 76 (2013). https://doi.org/10.1016/j.envpol.2012.08.011

9. Yu. N. Morgalev, I. A. Gosteva, T. G. Morgaleva, S. Yu. Morgalev, E. V. Kostenko, and B. A. Kudryavtsev, Nanotechnol. Russ. **13**, 311 (2018).

10. E. J. Parka, G. H. Leeb, C. Yoonc, et al., J. Appl. Toxicol. **36**, 424 (2016). doi org/ https://doi.org/10.1002/jat.3233

11. A. L. di Virgilio, M. Reigosa, and M. F. de Mele, J. Biomed. Mater. Res. A **92**, 80 (2010). https://doi.org/10.1002/jbm.a.32339

12. E. Radziun, J. Dudkiewicz-Wilczynska, I. Ksiaek, et al., Toxicol. Vitro **25**, 1694 (2011).

13. Z. M. Song, H. Tang, X. Deng, et al., J. Nanosci. Nanotechnol. **17**, 2881 (2017). https://doi.org/10.1166/jnn.2017.13056

14. E. Dong, Y. Wang, S. T. Yang, et al., J. Nanosci. Nanotechnol. **11**, 7848 (2011). https://doi.org/10.1166/jnn.2011.4748

15. I. V. Shugalei, A. V. Garabadzhiu, M. A. Ilyushin, and A. M. Sudarikov, Ekol. Khim. **21**, 172 (2012).

16. A. S. Cardwell, W. J. Adams, R. W. Gensemer, et al., Environ. Toxicol. Chem. **37**, 36 (2018). https://doi.org/10.1002/etc.3901

17. S. Pakrashi, S. Dalai, A. Humayun, et al., PLOS One **8** (9), e74003 (2013). https://doi.org/10.1371/journal.pone.0074003

18. N. Musee, P. J. Oberholster, L. Sikhwivhilu, and A. M. Botha, Chemosphere **81**, 1196 (2010). https://doi.org/10.1016/j.chemosphere.2010.09.040

19. P. V. Vidya and K. C. Chitra, Int. J. Fisher. Aquat. Stud. **3**, 13 (2018).

20. J. G. Coleman, D. R. Johnson, J. K. Stanley, et al., Environ. Toxicol. Chem. **29**, 1575 (2010). https://doi.org/10.1002/etc.196

21. T. Stadler, M. Buteler, D. K. Weaver, and S. Sofie, J. Stored Prod. Res. **48**, 81 (2012). https://doi.org/10.1016/j.jspr.2011.09.004

22. GOST (State Standard) No. 31674-2012: Feed, compound feed, compound feed raw materials. Methods for determining general toxicity (2012; 2016).

23. M. Kryuchkova, A. Danilushkina, Y. Lvov, and R. Fakhrullin, Environ. Sci.: Nano **3**, 442 (2016). https://doi.org/10.1039/c5en00201j

24. G. I. Fakhrullina, F. S. Akhatova, Y. M. Lvov, and R. F. Fakhrullin, Environ. Sci.: Nano **2**, 54 (2015). https://doi.org/10.1039/C4EN00135D

25. K. Li, Y. Chen, W. Zhang, et al., Chem. Res. Toxicol. **25**, 1675 (2012). https://doi.org/10.1021/tx300151y

26. I. Gosteva, Yu. Morgalev, T. Morgaleva, and S. Morgalev, IOP Conf. Ser.: Mater. Sci. Eng. **98**, 012007 (2015). https://doi.org/10.1088/1757-899X/98/1/012007

27. M. A. Gatoo, S. Naseem, M. Y. Arfat, et al., BioMed Res. Int. **8**, 498420 (2014). https://doi.org/10.1155/2014/498420

28. N. V. Zaitseva, M. A. Zemlyanova, M. S. Stepankov, and A. M. Ignatova, Ekol. Cheloveka, No. 5, **9** (2018). https://doi.org/10.33396/1728-0869-2018-5-9-15