AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

Comparative evaluation of the toxicity of iron and its oxides nanoparticles using *Stylonchia mytilus*

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Abstract. Indeed, about 33 tons of engineered nanomaterials are being released into the hydrosphere of the earth every year. Nanoparticles are increasingly being utilized for commercial purposes, however, the positive and negative properties of such materials on the environment and humans are still to be fully elucidated. However, the toxicity of nanomaterials to the environment and their effect on hydrobionts needs to be further studied. Due to the increasing amount of nanoparticles in the surrounding, it is essential to study how these are accumulated in water invertebrates. The aim of this study was to determine the toxicity of different doses of iron nanoparticles and its oxides and the utilisation of *Stylonychia mytilus* as a model organism. Fresh water *S. mytilus* (wild strain) was utilized in the experiments during its exponential growth phase. The tested functions were survival and quantity (biomass). During researches Fe3O4 (I), Fe3O4 (II), and Fe (I) nanoparticles was used, composition of FeCo. Analysis of concentration effects revealed that the nanoparticles of Fe (I) stimulated the maximum toxic effect which observed at concentrations up to 4 x 10^{-5} M on the cells of protozoa. In all other dilutions up to 6 x 10^{-6} M the number of surviving cells was gradually increased (from 40 to 95%). Toxic effect of iron oxides also is saved up to 9 x 10^{-5} M. With each subsequent dilution the number of living cells increased. The conducted experiments show that *S. mytilus* is highly susceptible to heavy metals, as shown by the nanoparticles of iron. Thus, protozoa can be utilized as test organisms to determine the heavy metal toxicity of environments.

Key Words: iron nanoparticles, infusoria, ciliate protozoan, toxicity, free-radical oxidation, cell membrane.

Аннотация. В гидросферу Земли поступает около 33 тонн техногенных наноматериалов. Вследствие чего, область применения нанотехнологий быстро расширяется, при этом не до конца известны все свойства наноматериалов, их положительные и отрицательные стороны. Так же до конца не изучен вопрос токсичности наноматериалов, но в настоящий момент – это одна из приоритетных областей изучения, так как наноматериалы широко применяются, и напрямую взаимодействуют с организмом человека. Поведение наноматериалов в окружающей среде и их влияние на гидробионтов изучено далеко неполно и требует пристального внимания, поскольку их поступление в окружающую среду увеличиваться, особенно вопросы биоаккумуляция техногенных наночастиц в водных беспозвоночных. Целью данного исследования является оценка острой токсичности различных доз наночастиц железа и его оксидов, с использованием в качестве тест-объекта *Stylonychia mytilus.* В качестве тест-объекта использовали культуру клеток пресноводной инфузории *S. mytilus* (дикий штамм) в фазе экспоненциального роста. В число исследуемых тест-функций вошли: выживаемость, численность (биомасса). В исследованиях использовали наночастицы Fe3O4 (I), Fe3O4 (II), Fe (I), наносистема FeCo. Анализ концентрационных воздействий показал, что наночастицы Fe (I) вызывают максимальный токсических эффект, который наблюдался при концентрации до 4 x 10^{-5} M на клетки простейших. Во всех остальных разведениях до 6 x 10^{-6} M число выживших клеток постепенно увеличивалось (от 40-95%). Токсичное действие оксидов железа также сохранялось до 9 x 10^{-5} M. С каждым последующим разведением число живых клеток увеличивалось. Проведенные эксперименты свидетельствуют о том, что высокая чувствительность одноклеточных микроорганизмов к токсическому действию тяжелых металлов (на примере наночастиц железа) позволяет использовать инфузории в биологических тестах с целью оценки токсичности сред на наличие и концентрации тяжелых металлов.

Ключевые слова: наночастицы железа, инфузории, *Stylonychia mytilus*, токсичность, окисление свободных радикалов, клеточная мембрана.

Rezumat. Hidrosfera pământului primește anual aproximativ 33 de tone de nanomateriale artificiale. Ca urmare, aplicarea nanotehnologiei se extinde rapid, în timp ce nu sunt pe deplin cunoscute toate proprietatile nanomaterialelor, respectiv laturile lor pozitive și negative. Problema toxicității nanomaterialelor este una de actualitate în condițiile în care nu este elucidată pe deplin interacțiunea directă cu mediul înconjurător și nu în ultimul rand cu organismul uman. Din acest motiv actualmente aceste demersuri constituie una dintre domeniile prioritare de studiu. Comportamentul nanomaterialelor în mediu și efectul lor asupra organismelor acvatice studiate este departe de a fi completă, și necesită o atenție deosebită, datorită pătrunderii crescânde a acestora în mediul înconjurător. Cele mai stringente ar fi elucidarea interacțiunii acestor materiale vis-a-vis de specia umană. Scopul acestui studiu este de a evalua toxicitatea acută a diferitelor concentrații de nanoparticule de fier și oxizi de fier, utilizând ca și organism model *Stylonychia mytilus*. Ca și material biologic de testare s-a utilizat o cultură celulară de ciliate apă dulce *S. mytilus* (tipul sălbatic) aflată în faza de creștere exponențială. Printre funcțiile studiate au fost incluse procentul de supraviețuire și cantitatea (de biomasă). În studiu au fost folosite nanoparticulele: Fe3O4 (I), Fe3O4 (II), Fe (I), și componenta FeCo. Studiul a evidențiat efecte toxice maxime a nanoparticulei Fe (I) în celula protozoarei la o concentrație de până la 4 x 10^{-5} M. In toate celelalte diluții, până la 6 x 10^{-6} M, numărul de celule supraviețuitoare a crescut treptat (de la 40 la 95%). Efectul toxic a oxizilor de fier, este neglijabil până la concentrația de 9 x 10^{-5} M. Cu fiecare diluție ulterioară numărului de celule vii a crescut. Experimentele arată că sensibilitatea ridicată a microorganismelor unicelulare la efectele toxice ale metalelor grele (de exemplu nanoparticule de fier), recomandă protozoarele ciliate pentru analize biologice în vederea evaluării mediilor toxice în prezența metalelor grele.
Cuvinte cheie: nanoparticule de fier, ciliate, protozoar ciliat, toxicitate, oxidarea radicalilor liberi, membrana celulară.

Introduction. Each year, new nanomaterials are being developed, being by now more than four thousand. This however, also results into an increasing amount of engineered nanoparticles being released into the ecosystem (Tretyakov 2007). Indeed, about 33 tons of engineered nanomaterials are being released into the hydrosphere of the earth (Kolesnichenko et al 2008). Nanoparticles are increasingly being utilised for commercial purposes, however, the positive and negative properties of such materials on the environment and humans are still to be fully elucidated (Piotrovski & Kiselev 2006; Nel et al 2006).

Metal nanoparticles, their oxides and mixes are commonly utilised in molecules purification, to deliver drugs into tissues and cells (Piotrovski & Kiselev 2006) and for diseases diagnosis (Keller et al 2013). However, even though iron nanoparticles are broadly utilized, its compounds cause colloid instability upon being delivered into biological systems. Additionally, the particles bind non-specifically to cell receptors and result in toxicity (Du et al 2013). Recently, many articles on the toxicity of several nanomaterials were published. However, most of them are based on studies of animal models. Nevertheless, many of the defense and adaptation reactions of any organism to an unfavorable factor are the same, independently of the species (Zhu et al 2006; Haq et al 2000). Unicellular protozoa have a large contact surface with the surroundings, relative to their size. Upon the introduction of a toxic molecule, they are immediately affected and react with a complex of actions: chemotaxis, reversion of cilial activity and rate of reproduction. In addition, many hydrobionts feed through filtration that increases the chances of accumulation of the toxic particles within the organism and this resulting in an increased effect of the pollutant on the cell (Zhu et al 2006). The ecological risks and methodologies to test the toxicity of nanomaterials utilizing hydrobionts were described in recent publications (Haq et al 2000). For example, the utilization of *Euglena*, *Vorticella* and *Stylonychia* protozoa to test the toxicity of industrial waste waters was described (Haq et al 2000). However, the toxicity of nanomaterials to the environment and their effect on hydrobionts needs to be further studied. Due to the increasing amount of nanoparticles in the surroundings, it is essential to study how these are accumulated in water invertebrates (Baun et al 2008). The aim of this study was to determine the toxicity of different doses of iron nanoparticles and its oxides and the utilization of *Stylonychia mytilus* as a model organism.

Material and Method. Fresh water *S. mytilus* (wild strain) was utilized 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 diluted 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 utilizing 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 utilized as a control for all experiments.

The nanoparticles utilized in the study are described in Table 1.

Table 1

Characteristics of the utilized nanoparticles

Name	*Size (nm)*	*Phase and chemical composition*	*Production method*	*Surface (m^2/g)*	*Producer*
Fe_3O_4 (I)	65	Fe3O4 at least 99% wt., about 1% of the mass. - adsorbed gases: CH_4, CO_2, O_2, N_2	Method of electric explosion of a conductor in air	10	«Advanced powder technologies», Russia
Fe_3O_4 (II)	65	Fe3O4 99 % of mass.	Chemical	20	«Advanced powder technologies», Russia
Fe (I)	90	Metallic iron (not less than 99.8% of the mass.) and sorbed gases: CH_4, CO_2, Ar, N_2.	Method of electric explosion of a conductor in air	7.7	«Advanced powder technologies», Russia
FeCo	62.5	70% Fe, 30% Co	Gas-phase	8.2	«Advanced powder technologies», Russia

Nanoparticles preparation was performed 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 x 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. The analysis of the data revealed different effects of forms of iron nanoparticle to culture cells of infusoria (Table 2).

Table 2

Effects of forms of iron nanoparticles in cell culture *Stylonchia mytilus*

Nanoparticle	Concentration, M																			
	3.2	1.6	0.8	0.4	0.2	0.1	0.05	0.025	0.0125	0.00625	0.003	0.0015	0.00078	0.00039	0.00019	$9x10^{-5}$	$4x10^{-5}$	$2x10^{-5}$	$1x10^{-5}$	$6x10^{-6}$
Time of exposure: 10 min																				
Fe I	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (I)	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (II)	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
FeCo	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Time of exposure: 60 min																				
Fe I	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (I)	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (II)	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
FeCo	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Time of exposure: 360 min																				
Fe I	Tox	Tox	Tox	Tox	Tox	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (I)	Tox	Tox	Tox	Tox	Tox	Tox	Tox	LOEC	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (II)	Tox	Tox	Tox	Tox	Tox	Tox	LOEC	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
FeCo	Tox	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Time of exposure: 720 min																				
Fe I	Tox	Tox	Tox	Tox	Tox	Tox	Tox	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (I)	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	LOEC	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (II)	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	LOEC	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
FeCo	Tox	Tox	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Time of exposure: 1,440 min																				
Fe I	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Fe3O4 (I)	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	LOEC	LC50	NOEC	NOEC	NOEC	NOEC
Fe3O4 (II)	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
FeCo	Tox	Tox	Tox	Tox	Tox	LOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC

Tox – the concentration causing 0-39 % survival of the subjects; LC50 – the concentration causing 50% survival of the subjects; LOEC – the concentration causing 40-69 % survival of the subjects; NOEC – the concentration causing 70-100 % survival of the subjects (Jackson et al 2013).

Table 3 shows the morphological changes of cells (flocculation, rupture, atrophy) depending on the duration of incubation of cell cultures of *S. mytilus* with nanoparticles.

Table 3

Morphological changes of cells *Stylonchia mytilus* on the background of toxic effects of nanoparticles

Time of incubation (h)	*Cell morphology*			
	Fe (I)	*Fe_3O_4 (I)*	*Fe_3O_4 (II)*	*FeCo*
1	Normal, flocculation	Normal, flocculation	Normal, flocculation	Normal, flocculation
2	Flocculation	Flocculation, rupture	Flocculation, rupture	Flocculation
6	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy
12	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy
24	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy	Flocculation, rupture, atrophy

The results show no influence of the nanoparticles during the first hour of incubation, and the complete destruction of the membrane starting at 6^{th} hours of exposure.

All the tested particles had a similar effect on the cells, causing death of the cells after 24 hours (Figure 1. Effects of forms of iron nanoparticles in cell culture *Stylonchia mytilus* summarizes how different iron nanoparticles affected *S. mytilus*. The death was caused because of the destruction of the plasma membrane due to an intensive free-radical oxidation.

Analysis of concentration effects revealed that the nanoparticles of Fe (I) stimulated the maximum toxic effect which observed at concentrations up to 4×10^{-5} M on the cells of protozoa. In all other dilutions up to 6×10^{-6} M the number of surviving cells was gradually increased (from 40 to 95%). Toxic effect of iron oxides also saved up to 9×10^{-5} M. With each subsequent dilution of the number of living cells increased.

The nanosystem FeCo had less toxicity, full cell death was observed after 24 hours at a concentration of 0.05 M, in the other concentrations the toxic effects have been not identified.

The morphology of *S. mytilus* changed upon particle introduction, with a noticeable disruption of the plasma membrane (Figure 1 B & D). The changes in the cell morphology started during the second hour of particle treatment. Part of the cell population showed flocculation. Additionally, some of the cells treated with Fe_3O_4 (I) and (II) had sign of membrane rupture. After 6, 12 and 24 hours all cells treated with the particles showed flocculation, membrane rupture and atrophy. The obtained images of *S. mytilus* suggest that initially the particles bind to the cell membrane (Figure 1C) and are then internalized by endocytosis. Subsequently, they are transported into lysosomes and bind to them, disrupting their membrane. This releases the degradation enzymes within the organelle and thus the cell starts «digesting» itself from within. During the first 6 hours of treatment, the particles were located only at the periphery of the cells (Figure 1C). After 12 hours, they were distributed homogeneously within the cells.

S. mytilus chemotaxis could also be noticed in the cultures. Indeed, most of the dead cells were located at the perimeter, away from the highest concentration of nanoparticles.

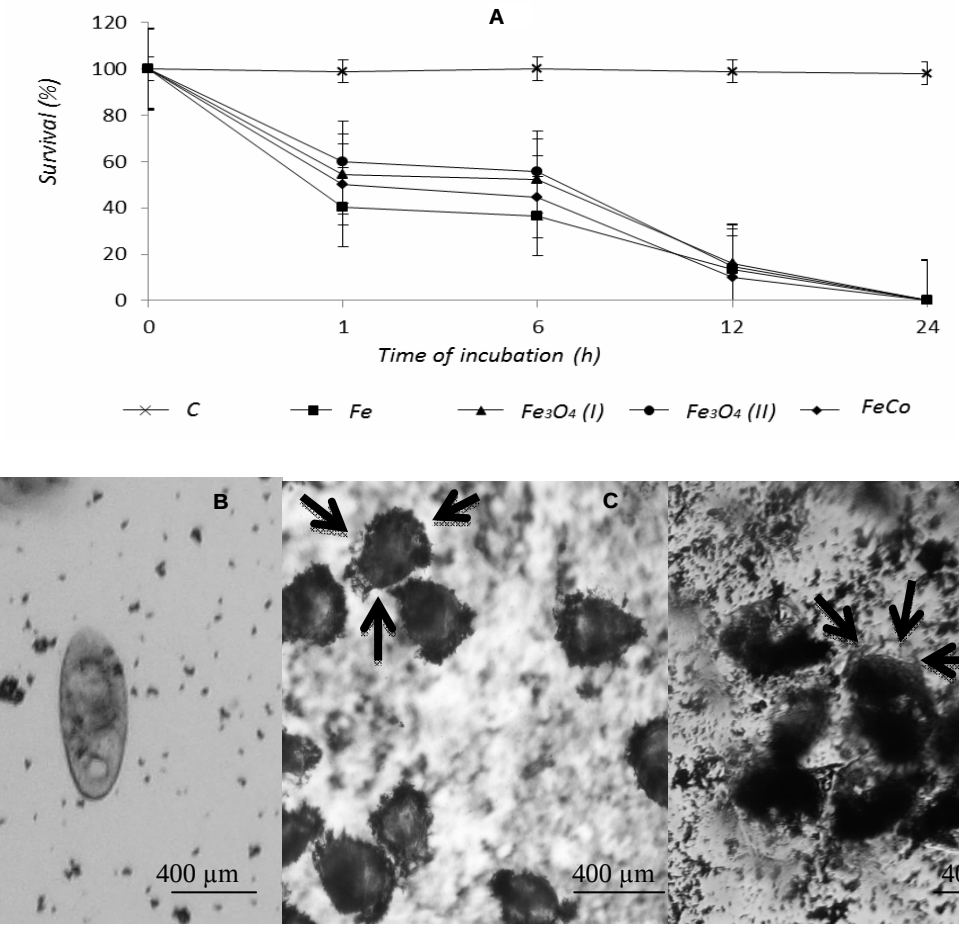

Figure 1. Effects of forms of iron nanoparticles in cell culture *Stylonchia mytilus*. A) Changes in survival (mean±SD) of *S. mytilus* through time upon treatment with different iron particles; B) *S. mytilus* one hour after the addition of the iron particles; C) *S. mytilus* 6 hours after the addition of the iron particles; D) *S. mytilus* 24 hours after the addition of the iron particles. The arrows show disruptions of the membrane. Scale bar 400 µm.

Our results are in accordance whit previous reports, which emphasized that the toxicity of iron oxides and mixes nanoparticles depends on the concentration and time of exposure (Zhu et al 2006). Due to the internalization of the particles inside the cell, free radicals are formed, which lead to oxidative stress. The «oxidative explosion» is one of the earliest reactions to infection (Zhang et al 2007). Before, reactive oxygen species (ROS) were considered highly toxic molecules that inhibited the development of pathogens. However, recently a new function of them was determined, to trigger the transcription of genes involved in defense mechanisms of the cell. For instance, an increase in ROS levels within a cell triggers the oxidation of plasma membrane lipids that results into changes in its structure and consequently increased permeability (Leroueil et al 2008). Such cellular reactions can happen before or after the infiltration of the particles and also as an answer to phagocytosis, when the cell cannot uptake a particle due to its size or shape (Nowack 2009). This is due to the membrane ability to selectively transport ions, molecules and nanoparticles, in order to keep homeostasis with the surroundings. However, such property makes the cells vulnerable to the detrimental action of nanoparticles (Wiesner et al 2008).

The physical process of translocation of iron nanoparticles through the membrane is due, primarily, to the relevant dimensions and properties of nanomaterials. Data includes membrane activity (Stadtman 1990; Abalenihina et al 2012), transport chain

(Bottini et al 2006), protein conformation (Cui et al 2005) and aggregation (Kagan et al 2006).

The conducted study showed that the particles localize mostly in lysosomes. The cell is either trying to digest the particles, or secrete them back into the surroundings.

Conclusions. There is voluminous data on the study of oxidative stress, as this is one of the first indicators of free radical damage. Such studies show that the oxidation of proteins caused by the hydroxide anion occurs with the presence of metals of variable valence (Aust et al 1985; Stadtman 1990). This is called metal-catalysed oxidation and is considered one of the post-translational modifications of proteins. The oxidation of the protein amino acids occurs in the presence of metals of variable valence (iron, copper), oxygen, and H_2O_2. The oxidation affects the part of the protein that binds to the metal. For example, copper nanoparticles increase the oxidative processes of the proteins in the rat thymus. Such condition was accompanied by a change in metabolism and on the possibility of renewal of the tissue proteins (Abalenihina et al 2012). Carbon nanotubes have a similar effect, however, the mechanism of their toxicity is yet to be found. Based on studies on T-lymphocytes (Bottini et al 2006) and HEK293 cells (Cui et al 2005) it was hypothesised that the nanotubes induce apoptosis by accumulating ROS, while in the RAW264.7 cells strong oxidation stress was detected. Others suggest that the nanotubes cause molecular toxicity (Ding et al 2005).

The conducted experiments show that *S. mytilus* is highly susceptible to heavy metals, as shown by the nanoparticles of iron. Thus, protozoa can be utilized as test organisms to determine the heavy metal toxicity of specific environments.

Acknowledgements. This study was supported by the Russian Science Foundation, Russia, project no. 14-36-00023.

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Received: 16 May 2015. Accepted: 18 June 2015. Published online: 22 June 2015.
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How to cite this article:
Kosyan D. B., Rusakova E. A., Miroshnikov S. A., Sizova E. A., Notova S. V., Yausheva E. V., Korotkova A. M., 2015 Comparative evaluation of the toxicity of iron and its oxides nanoparticles using *Stylonchia mytilus*. AACL Bioflux 8(3):453-460.