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Biological Activity of Nanocomposite Detoxicant in Biotest-Systems

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Synthetic nanoparticles (NPs) are widely recommended as effective structural components of agents for remediation of environments. The choice of an application dose of nanosorbents is based usually mainly on the sorption properties. Despite of a growing understanding that synthetic NPs should be evaluated for their potential environmental hazard prior their use in products and release into the environment, there are currently few data on the toxicity of nanomaterials to environmentally relevant species. Responses of living organisms in standard biotests under controlled conditions remain the most important part of the safety assessment in ecotoxicological purposes. We recommend the battery tests organisms which represent the main trophic ecosystem levels to establish the safety dose for elaborated nanosorbent of toxic substances.This work summarizes the major features of a magnetoactive nanocomposite sorbent of uranyl and cadmium ions synthesized based on magnetite and humic acids. An assessment of ecotoxicity and biological activity of nanocomposite using four bioassay systems (algae, paramecium, mustard seed, and bull spermatozoa) allowed us to determine concentration limits for application of this detoxicant.

Key words: magnetite, humic acid, sorbent, detoxicant, remediation, biotesting

INTRODUCTION

Currently there has been an increase in the scope of application of nano-materials in various fields of ecology. In particular, of great interest is the use of nanomaterials for creating effective sorbents of toxic substances that could be used o offset adverse effects on the environment (Ponder et al., 2000; Tartaj, Serna, 2003; Savage and Diallo, 2005; Diallo et al., 2005; NanoAction, 2007; Jorobekova, Kydralieva, 2010; European Technology, 2011).

Nanotechnologies allow a more complete characterization of new products, but still little is known about their interaction with living organisms. At the same time, these issues are most important in the development of safe nanosystems in all spheres, including the use of sorbents of toxic substances. Testing of the biosafety and biological activity of materials for remediation technologies is an integral part of environmental protection measures. There is a need for methods of toxicological control of manufactured nanomaterials, the number of nanomaterials already exceeds 1800. Russia has enacted a number of decrees and regulations pertaining to nanomaterial toxicology (e.g., Concept of toxicological studies … 2007; Toxicological assessment of the safety of nanomaterials, 2009). Despite the great efforts in this area, there is yet no detailed description and classification of the responses of living organisms to engineered materials (Lystsov et al.,, 2007; Andreev et al., 2008, 2009 Masycheva, et al., 2009; Radilov, 2009; Brayner, 2007; Nohynek et al., 2008; Nasir, 2010; Hu and Gao, 2010; and others). Toxicological testing performed both after the application of nanostructured detoxicants and at various stages of their production should ensure the reliability of the risk assessments and estimates of the consequences of chemical pollution of natural environments.

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The use of nanocomposite materials as selective and amenable mineral and polymer sorbents for binding and recycling of radionuclides and heavy metal ions seems very promising (Pomogailo and Kestelman, 2005). It is advisable to create this type of nanocomposite sorbents based on nanoparticles of magnetite and green "chemicals" - humic substances. The use of humic substances is based on their unique biological properties, including biocompatibility, stability and multifunctionality, which, in particular, make possible binding of hydrophobic organic compounds or metal ions through physical sorption and ion exchange mechanisms (Zhorobekova, 1986; Pomogailo, Rosenberg, 2000). The main structural units of humic acid (HA) molecules are the aromatic core and peripheral carboxyl functional groups: carboxyl, phenol hydroxyl, methoxyl and carbonyl groups, which are responsible for their high reactivity and high (up to 1 mmol/g) sorption capacity by complexation reactions and formation of chelate structures. The prospects of practical use of humic substances and their derivatives as sorbents are determined by large resources of humus-containing materials, which include brown coal, peat, sapropel etc. (Choppin, 1999).

In the present work, we used a small proportion of the sorption capacity (less than 10%) of humic acids to bind magnetic nanoparticles. Such nanocomposites acquire magnetic properties that can be used in the method of magnetic separation of toxic wastes (Zhong et al., 2006; Gao et al., 2007). In this case, humic substances can, on the one hand, serve as effective stabilizers for magnetoactive metal nanoparticles, preventing the aggregation into large clumps and, on the other hand, retain their protective properties as absorbents of ecotoxicants (uranyl ion and ions of heavy metals).

We present the main toxicological characteristics of a new nanostructured composite, a sorbent for ecotoxicants, as well as results of the evaluation of its biological activity. Based on these data, which are based on toxicity tests in a variety of biotic test systems, we provide an estimate of the concentration limits of the use of these materials.

MATERIALS AND METHODS

Synthesis and physicochemical properties of nanocomposite

The humic acids (HA) used in this study were obtained from potassium humate (Powhumus, Humintech Ltd) by dialysis and were shown to have an ash content of 8.95%, COOH-group content of 4.2 mmol/g, and phenolic group content of 1.1 mmol/g. Highly dispersed magnetite (Fe_3O_4) was prepared using the Elmore reaction (Elmore, 1938), i.e. *in situ* coprecipitation salts of iron (II) and (III) in the presence of these HA in three ratios (Fe_3O_4/HA, wt %/wt%: 20/80; 50/50; 80/20).

Composition and structure of the obtained highly dispersed magnetite powders were characterized using Mössbauer spectroscopy (WISSEL, Co57(Rh)), XRD analysis (Philips X-pert, Cu-K_α), and transmission electronic microscopy (Hitachi H-7000; Fetisov *et al.*, 2010; Zaripova *et al.*, 2008). An ultrasound particle analyzer DT-1200 Quantachrome Dispersion Technology was used to estimate the size distribution in magnetite nanoparticle suspensions.

Analysis of sorption properties of nanocomposite

Adsorption of UO_2^{2+} and Cd^{2+} ions in the presence of HA alone or nanocomposite Fe_3O_4/HA was studied after the addition of aqueous solutions of $UO_2(NO_3)_2$ or $Cd(NO_3)_2$ to the sorbent suspension (5 g/L) using a range of 0.44-16.02 mmol/L. Equilibrium concentration of the uranyl and lead ions in the supernatant was determined with a photocolorimeter (KFK-2, Russia, λ_{max} = 655 nm) in the presence of arsenazo III and with an atomic absorption spectrophotometer (Pay

Unicam, USA), respectively. Sorption processes during interaction of metals with humic acids were described with the Langmuir isotherm:

$$Q/Q_{max} = K_{sorp}[M]/(1 + K_{sorp}[M]),$$

where Q is the quantity of adsorbate metal per unit mass of sorbent; Q_{max} is the maximum adsorption, K_{sorb} is the constant of adsorption, [M] is the equilibrium concentration of metal.

Biotesting methods

For ecotoxicological assessment, samples of the nanohybrid composite were used as water suspensions in the following concentrations (%): 0.001, 0.01, 0.1, 1.0. Water suspensions were prepared in distilled water or in the relevant culturing medium in accordance with the requirements of the standard biotesting methods. The Uspenskii-1 medium for the algae contained (g/L): KNO_3 – 0.025; $MgSO_4$ – 0.025; KH_2PO_4 –0.025; $Ca(NO_3)_2$ – 0.1; K_2CO_3 – 0.0345; Iron III citrate water ($C_6H_5O_7Fe \cdot nH_2O$) 0.002. The culture medium for bull spermatozoa contained glucose 40 g/L and sodium citrate 1.0 g/L.

Biological testing of $Fe_3O_4/HA_{20/80}$ was conducted using biotesting methods recommended for environmental control by the Ministry of Natural Resources and Environment of the Russian Federation The "battery" of biotests included standardized test-cultures of organisms of different taxonomic groups – algae, higher plants, protozoa, and mammalian cells.

Biotesting with the green protococcus alga *Scenedesmus quadricauda* (Turp.) Breb. used changes in the level of chlorophyll fluorescence and cell number of algae after 72 h of algal exposure in Uspenskii-1 medium without $Fe_3O_4/HA_{20/80}$ (control) and with added $Fe_3O_4/HA_{20/80}$ (treatment) in accordance with the method registered in the Russian Federal Register of Measurement Techniques – FR.1.39.2007.03223 (Zhmur, Orlov, 2007). Fluorescence parameters were measured with a Water-PAM (Walz, Germany) pulse fluorometer. Cell number in suspensions of *Scenedesmus* was measured by direct counting under the microscope in a Goryaev chamber. Biological activity of the preparations was tested on seeds of the higher plant white mustard *Sinapis alba* L. by the modified Persoone et al. (2006) method using the Phytotoxkit for seed germination and early root growth (www.microbiotest.com; Lisovitskaya, Terekhova, 2010). The length of roots and sprouts in mustard seedlings was compared after 96 h exposure at 24^{o}C in plastic containers maintained in darkness.

A biotesting system with protozoa was used to compare survival of *Paramecium caudatum* Ehrenberg after a 24 h incubation with and without nanocomposite sorbent, according to method FR.1.39.2006.02506 (Rakhleeva, Terekhova, 2006). The animals were counted in microaquaria under a stereoscopic microscope MBS-2 LZOS, Russia, 2008). Only the two lowest nanocomposite doses of the nanoparticle–HA solution (0.001 % and 0.01 %) could be evaluated in the paramecium test system because both the 0.1 % and the 1.0 % solutions were too dark to distinguish the paramecia from the surrounding solution.

Toxicity of the decreased preparation with mammalian cell culture was determined by evaluating mobility *in vitro* of bull (*Bos taurus taurus* L.) spermatozoa obtained frozen in nitrogen from the Institute of Medical Technique (Moscow, Russia) generated according to Russian State standard GOST 26030-83. The sperm were defrosted at 40+1.5 ^{o}C and used in the original culture medium. The sperm with $Fe_3O_4/HA_{20/80}$ were co-incubated for 4-5 hr at 27 ^{o}C as prescribed by FR.1.31.2009.06301 using a video image analyzer AT-05 (Eskov et al., 2009). The effect of each concentration of nanocomposite preparation in each of the biotests was expressed as the percentage of difference with the control. The effective concentrations (low effect levels) were evaluated.

RESULTS AND DISCUSSION

According to the X-ray phase analysis, Mössbauer spectroscopy, and electronic microscopy, particle size for $Fe_3O_4/HA_{20/80}$ nanocomposite averaged to 7-10 nm (Zaripova et al., 2008). All of the methods agree about the particle size with little differences. According to ultrasonic spectroscopy, particle size distribution depends on the ratio of the precursors, magnetite and HA in the nanocomposite (Fig. 1). The average particle size by ultrasonic spectroscopy varied in the range of 10-100 nm depending on the ratios of the precursors.

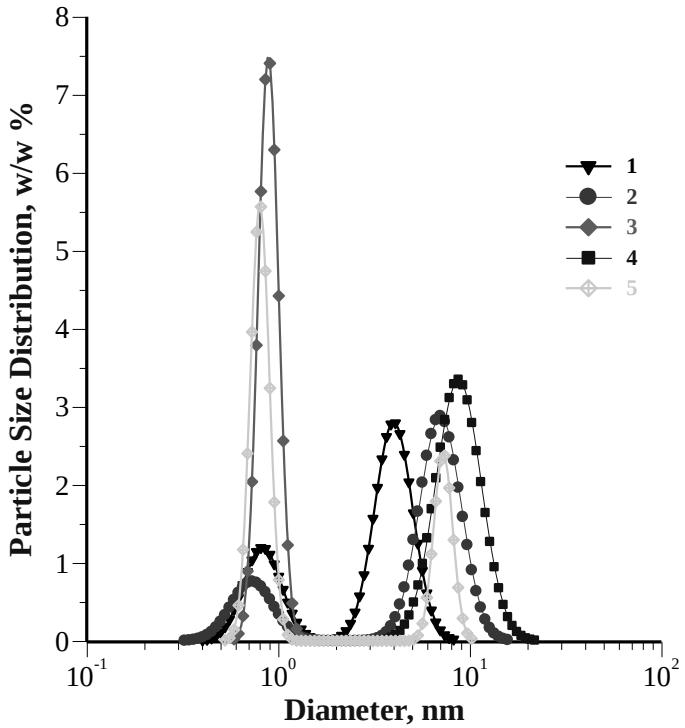

Figure 1: Particle size distribution of nanoparticles for different systems: (1) Fe_3O_4, (2) $Fe_3O_4/HA_{80/20}$; (3) $Fe_3O_4/HA_{50/50}$; (4) $Fe_3O_4/HA_{20/80}$; (5) $Fe_3O_4/HA_{20/80}$ (as prepared)

An increase in the average particle diameter and polydispersion was observed with an increase in magnetite concentration (from 10 to 100 nm and above). By changing the concentration of precursors in the system, we limited the growth of magnetite nanoparticles, thereby obtaining nanoparticles of the desired size. For sorption experiments a $Fe_3O_4/HA_{20/80}$ sample was chosen, which had the lowest particle size and a relatively narrow size distribution.

In the presence of polymeric macromolecules of HA, nanoparticles of magnetite were stabilized, the aggregative stability increased proportionally to the introduced HA concentration. Particle size of magnetite significantly decreased in the presence of the HA in all studied systems, probably due to capturing of metal particles by the HA organic macromolecule acting as a kind of "trap", i.e. the HA unit binding a nanoparticle during the nucleation stage, thus preventing aggregation. Moreover, the natural HA macromolecules provide for self-organization of the emerging metal-polymer structures leading to the size control of polymeric fragments at the level of the iron oxide nanoparticles (Pomogailo and Kestelman, 2005).

Sorption properties of the nanocomposite

Comparative analysis of sorption ability of the native HA and the $Fe_3O_4/HA_{20/80}$ nanocomposite showed that the materials studied are characterized by a high sorption capacity for uranyl ions, which is due to the formation of relatively stable actinide complexes with HA in a weak acid or neutral media as indicated by the relatively high values of their stability constants (log β varies from 5.85 to 11 for UO_2^{2+}-HA complexes; Hsi and Langmuir, 1985; Pompe *et al.*, 1998).

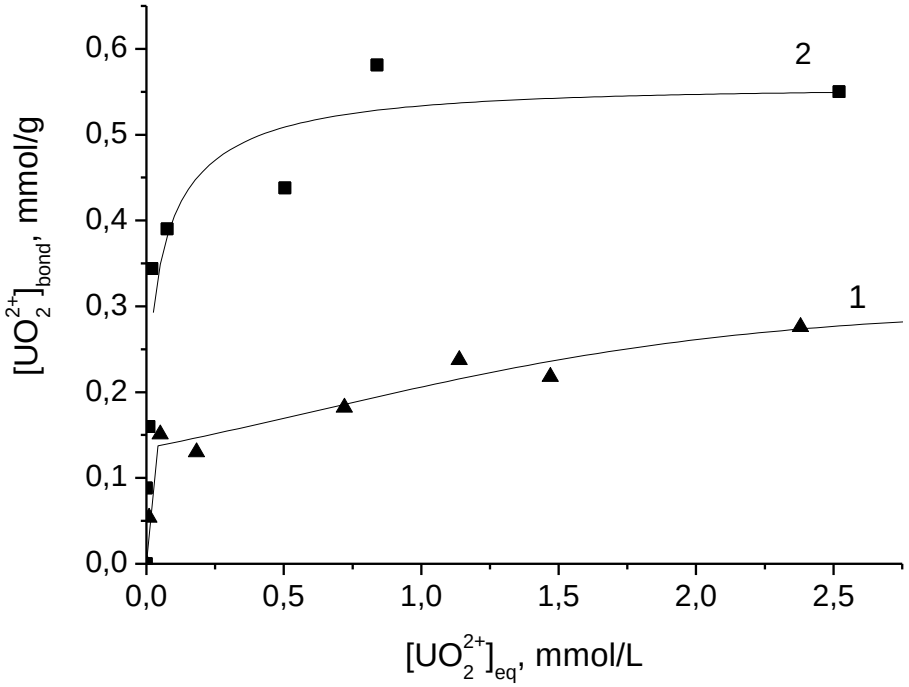

Figure 2: Adsorption isotherms of UO_2^{2+} ions by the humic acids (1) and the Fe_3O_4/HA nanocomposite (2)

Adsorption parameters were calculated based on Langmuir sorption isotherms using linearized coordinates (Fig. 2). For the UO_2^{2+}-HA system we determined the sorption constant $K_{sorp.}$ = (4.1±1.6) 10^4 1/M, Q_{max} = 0.3±0.05 mmol/g, and for the UO_2^{2+}-Fe_3O_4/HA system the sorption constant was $K_{sorp.}$ = (3.5±1.4) $10^4$10/1M, Q_{Max} = 0.56±0.02 mmol/g. The data showed an increase in UO_2^{2+} ions sorption by the nanocomposite as compared to the HA alone. However, the nature of UO_2^{2+} ions sorption on mineral oxides can significantly vary depending on experimental conditions (pH, ionic strength and the presence and concentrations of humic substances; Missana *et al.*, 2003; Illes and Tombacz, 2006). For systems studied in this work, where magnetite particles stabilized by HA macromolecules form a stable colloidal dispersion in a wide pH range (Illes and Tombacz, 2006), the high sorption capacity of the nanocomposite for uranyl ions, may be related to the high dispersion and nanostructure nature of the magnetoactive sorbent. According to literature data, the values of the specific surface for the HA, magnetite nanoparticles, and nanocomposite increase in the sequence HA<Fe_3O_4<Fe_3O_4/HA and equal to 42, 62 and 64 m^2/g, respectively (Alvarez-Puebla *et al.*, 2005).

Both the HA and the Fe_3O_4/HA nanocomposite manifest similar sorption properties with respect to heavy metals. In particular, for adsorption of Cd^{2+} ions onto the Fe_3O_4/HA nanocomposite, the following parameters were obtained: $K_{sorp.}$ = 760 1/M, Q_{max} = 0.96 mmol/g.

Assessment of the competing influence of Mg^{2+} ions on the sorption of uranyl ions on the surface of the nanocomposite has shown that even 100x excess of ions of the alkaline-earth metal Mg^{2+} had no significant impact on sorption of UO_2^{2+} ions. The addition of magnesium ions to the solution reduced the sorption of uranyl ions from 81.5 to 79.6%. The complexing capacity of the nanocomposite to uranyl ions is higher than that for cadmium ions, which may mean a greater stability of the HA-UO_2^{2+} complex and selectivity of this type of binding.

Toxicological activity of the magnetoactive nanohybrid composite ($Fe_3O_4/HA_{20/80}$)

Biotesting helps to assess the toxicological activity of the samples (Zhmur, 1997). Nanoparticles, depending on their characteristics, concentration and interaction with other molecules can cause a wide range of cellular responses. It is known that nanomaterials with different properties can induce cellular damage, but they can also cause favorable cell response leading to increased production of energy or cell growth (Yakovlev, Stehnina, 2007; Terekhova *et al.*, 2009; Tutelyan, 2009; Amanda *et al.*, 2004, and other). Based on the relationships between the characteristics of nanomaterials and their potential toxic effect, we attempted to change the harmful properties of nanoparticles while leaving intact catalytically valuable features for industrial applications.

To increase the safety and reduce bioreactivity, some nanoparticles are covered with special substances, detergents and polymers. The active surface of magnetoactive nanoparticles of metals used in our work for the preparation of a sorbent was stabilized with natural polymers, humic acids. Biotesting techniques were used to demonstrate the lack of toxicity of the Fe_3O_4/HA composite, leaving the high sorption capacity of the humic acids and the magnetically alternative properties of the magnetic nanoparticles. The biotesting of the nanocomposite sorbent revealed that different biotest systems showed variable sensitivity to the effects of this preparation. Microalga *Scenedesmus quadricauda*, as compared to other test-cultures proved to be the most sensitive to the presence of the Fe_3O_4/HA composite (Fig. 3). Growth inhibition of *Scenedesmus* cells was observed already at the minimum concentration tested, 0.001%. The lowest concentration that caused a decrease in parameters of fluorescence was higher, 0.01%.

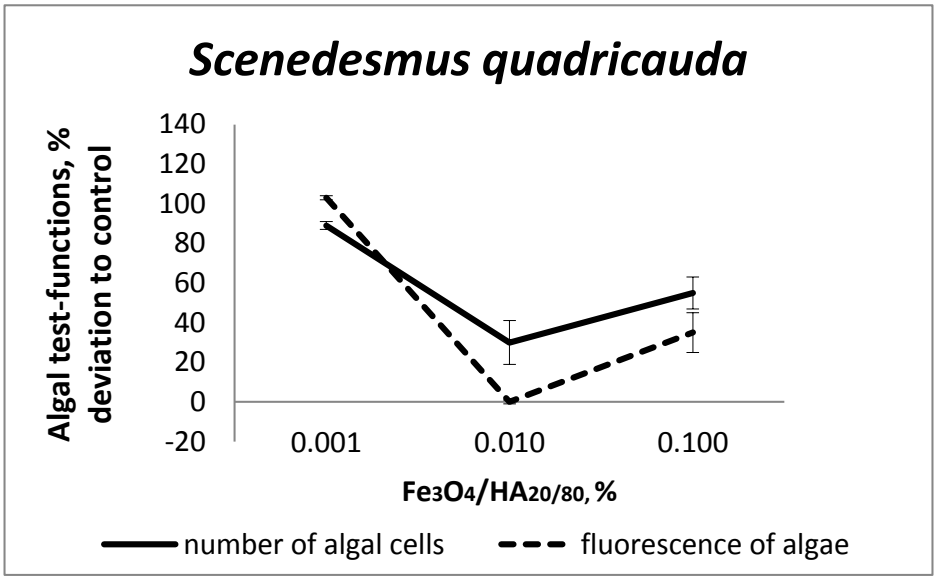

Figure 3: The effect of $Fe_3O_4/HA_{20/80}$ on changes in the increase of cell number and fluorescence intensity in cells of green alga *Scenedesmus quadricauda.*

A similarly sensitive test system was the plant root growth test. The root development in sprouts of white mustard *Sinapis alba* was noticeably stimulated by low concentrations of nanocomposite (0.001% and 0.01 %), while 0.1% reduced plant root growth to approximately 80% of control, and 1.0% completely blocked all sprouting.

The paramecia may have been the least sensitive to the nanoparticles-РΦ composite compared to the other test systems. Concentrations of 0.001% and 0.01% of the Fe_3O_4/HA composite were non-toxic to the culture of infuzoria *Paramecium caudatum*. There was even some stimulation of paramecium development; the number of treated individuals exceeded control at both concentrations by 20%. This growth stimulation may be due to the humic acid increasing the nutrient value of the solutions for the paramecia. Alternatively, the micro-electromagnetic fields induced by the magnetite nanoparticles could have stimulated the paramecium growth. As indicated in methods, no results could be obtained at the two highest composite concentrations.

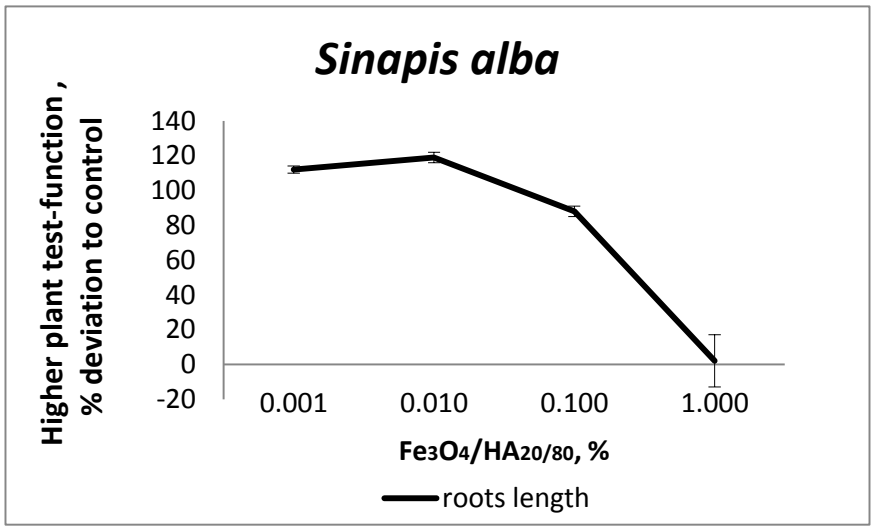

Figure 4: The effect $Fe_3O_4/HA_{20/80}$ on root growth of white mustard *Sinapis alba* seedlings.

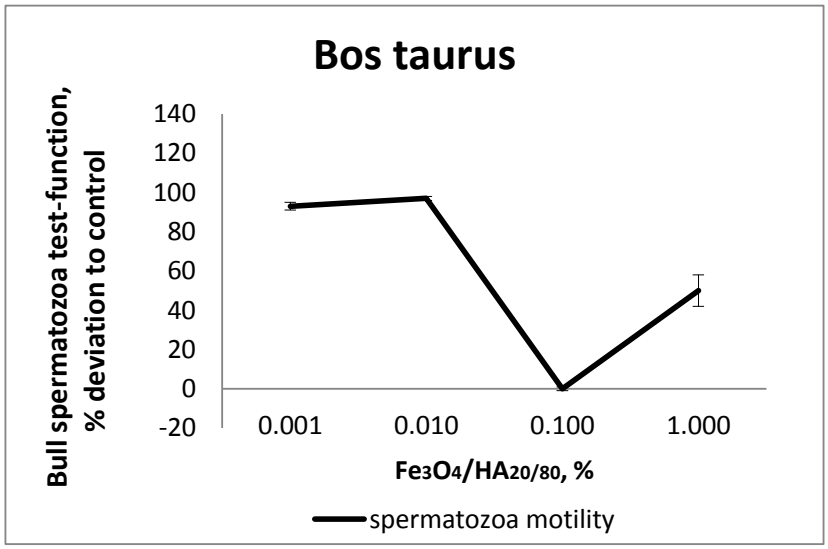

Figure 5: The effect of $Fe_3O_4/HA_{20/80}$ on motility changes on bull *Bos taurus* spermatozoa.

The nanoparticle-composite manifested a low adverse effect on the motile of bull

spermatozoa. An influence on the motility of bull sperm was found only at the highest concentration studied, 1.0% (Fig. 5). At 0.1% $Fe_3O_4/HA_{20/80}$ the motile of bull spermatozoa was like in control probe.

CONCLUSION

In conclusion, the effect of the nanocomposite on cells of various test organisms varied markedly. Based on biological response to the same concentration of the nanocomposite, species may be arranged in decreasing sensitivity as follows: *Scenedesmus quadricauda* > *Sinapis alba* > *Bos taurus taurus*, and the infusorians *Paramecium caudatum* exhibited no toxicity at the two lowest assessed concentrations. $Fe_3O_4/HA_{20/80}$ concentration in 0.001% was absolutely safe for all test organisms; the range from 0.001 to 0.01% was still safe for higher plants and bull spermatozoa but toxic for algal cells which appeared to be the most sensitive to $Fe_3O_4/HA_{20/80}$. Further concentration increases up to 0.1% and 1.0% were toxic for the whole battery of organisms. The summary of the no observed effect (NOEL), lowest observed effect levels (LOEL) and lowest observed adverse effect level (LOAEL) for each species and test are shown in Table 1.

Table 1. Analyzed $Fe_3O_4/HA_{20/80}$ effect levels to the different test-species

Species	Test parameter	NOEL	LOEL	LOAEL
Algae *Scenedesmus quadricauda*	cell number fluorescence	- 0.001	0.001 0.01	0.01 0.01
Higher plant *Sinapis alba*	root length	0.001	0.01	0.1
Infusorians *Paramecium caudatum*	Survival	0.01	-	-
Bull *Bos taurus taurus*	spermatozoan motility	0.01	0.1	0.1

This paper summarizes a set of bioassays using an iron-based magnetite nanoparticle-humic acid composite which has high sorption properties with respect to ions of heavy metals and uranyl ion. Experiments with this "battery" of four biotests showed that, in controlled chemical conditions, water suspensions of the preparation, can be used safely for biota only up to a certain concentration limit. Probably, in natural conditions the presence of organic material, especially of humic substances, may contribute to an increase in the permissible concentration limit and reduce the harmful effect of the nanocomposite sorbent. It is obvious that before applying such remediation under specific biotope conditions, the biosafety of the composite should be assessed with a similar biotesting battery.

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