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ORIGINAL RESEARCH PAPER

# ASSESSMENT OF POTENTIAL HAZARDS OF BIOGENIC METAL NANOPARTICLES TO AQUATIC PLANTS

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**SYNOPSIS**

The rapid development of nanotechnology is far ahead of nano assess the impact on the environment. Nowadays studies on the effect of nanomaterials, which can come in natural water pools with the waste water, are on special importance and their potential hazard to aquatic ecosystems must be assessed. The effect of colloidal solutions of biogenic metal nanoparticles, obtained from dispersion metals granules by impulse of electric current with amplitude 100-2000 A in water (at concentrations of Mn – 1,51 mg / l, Fe – 1,32 mg / l, Cu – 0,75 mg / l, Zn – 0, 89 mg / l) on the morphology, growth, reproduction and cytophysiological processes of pleustophytes *Limnobium laevigatum* (Humb. & Bonpl. Ex Willd.) Heine and *Lemna minor* L.) has been studied. It is shown a stimulating effect of nanoparticles of Fe, Mn, Fe-Mn and an inhibiting effect of Zn, Cu, Zn-Cu and Ag. Comparative assessment of metal nanoparticles and metals in ionic form impact on the example of Cu and Zn was conducted. Ions $Zn^{2+}$ and $Cu^{2+}$ at low concentrations stimulated intracellular physiological processes, growth, development and reproduction of plants, while high concentrations inhibited this processes. It has been shown that the effect of Zn and Cu nanoparticles was similar to the effect of ultrahigh concentration of metal in ionic form.

## INTRODUCTION

Nowadays nanotechnology is a strategic direction of development of economic in most countries including Ukraine. However, the potential risk of using engineered nanoparticles, especially in the natural environment, which may conceal a potential danger to the ecosystems, is not always understood. Questions related to the behavior of nanomaterials in natural ecosystems and their effect on living organisms requires close attention and precise research. The use of metal nanoparticles in crop production is considered as an alternative to highly toxic pesticides, fungicides, herbicides and insecticides. It is believed that their use can significantly reduce the concentration of metals entering the natural ecosystems from agricultural lands and to reduce human impacts on them.

Moreover, some results (Yershov et al., 2001; Rico et al., 2011; Dimkpa et al., 2012; Atha et al., 2012) that suggest the toxicity of some metals nanoparticles, such as zinc, copper and silver to living organisms are obtained. Bogoslovska et al. (2009) showed that metal nanoparticles can penetrate with remarkable ease through the cell membranes, causing DNA damage and uncontrolled cell division, which is directly related to the growth and development of living organisms. According to Heinlaan et al. (2008) the presence of nanoparticles in natural water pools reduces the productivity of aquatic organisms, gives them a variety of physiological changes, behavioral disorders and increases mortality.

Today, there information on the mechanisms of penetration, accumulation and transformation of nanoparticles in cells and organs of aquatic plants is incomplete. Thus, penetration of biogenic metal nanoparticles in natural aquatic ecosystems can cause the most unexpected effects, such as the development of aquatic plant species composition and improvement of biodiversity of phyto- and zoocenoses, as well as reduce productivity of sensitive species or increase productivity of resistant species. That could lead to a change of the number and ratio of species and imbalance of natural aquatic ecosystems. The aim of present study was to clarify the features of impact of biogenic metal nanoparticles, compared with ions of metal, on the growth, morphology, productivity, and intracellular processes of hydromacrophyta and to estimate the potential risk of metal nanoparticles to aquatic plant communities.

## MATERIAL AND METHODS

The study involved two types of floating on the surface of water plants: *Lemna minor* L. - plant with oval fronds, diameter of 1.5-2.5 mm, representative of the family *Lemnaceae* and *Limnobium laevigatum* (Humb. & Bonpl. Ex Willd.) Heine - plant with 2-3 cm rounded glossy leaves, representative of the family *Hydrocharitaceae*. *Lemna* and *Limnobium* cultures were cultivated in 40-60 liters aquariums on a settled tap water under optimum conditions: illumination - 6,000 lux, water temperature - 18-22 ° C, pH 8.5. Plants were exposed for 14 days in the vessels of 0.5 liters on a settled tap water with the addition of non-ionic colloidal solutions of biogenic metal nanoparticles and their binary compositions or metal ions in controlled conditions (illumination 5,000 lux at 10 hours photoperiod, water temperature - 22-27° C, air temperature - 20-25° C).

Colloid solutions of metal nanoparticles which are obtained by dispersing of the granules of iron, copper, manganese, zinc and silver by pulses of electric current with an amplitude A 100-2000 in water were studied (Lopatko et al., 2009). The maximum size of the nanoparticles is 100 nm. The concentration of metal nanoparticles in the stock sollutions were as follows: Mn - 151 mg / l, Fe - 132 mg / l, Cu - 75 mg / l, Zn - 89 mg / l. Stock solutions of nanoparticles of biogenic metals were diluted with water in a ratio of 1:100. In the experimental compositions the dose of each metal was halved.

Experiment design included the following options: 1) control (settled tap water), 2) the individual nanoparticles of biogenic metals (Mn, Fe, Zn, Cu, Ag); 3) binary compositions of metal nanoparticles (Fe-Mn, Cu-Zn); 4) metal ions $Zn^{2+}$ (salt $ZnSO_4$) and $Cu^{2+}$ (salt $CuSO_4$). Maximum allowed concentrations for Zn and Cu are 0,01 mg/l and 0,001 mg/l respectively.

Number of fronds in *Lemna* was counted on the 3$^{d}$, 6$^{th}$, and 9$^{th}$ day, as doubling number of fronds is normal every three days.

Determination of morphometric parameters: the mass of plants, number of leaves, number of roots, the number of individuals and newly formed subsidiary plants in *Limnobium* was carried out on the 1$^{st}$, 7$^{th}$ and 14$^{th}$ day.

Alteration of the intensity and degree of staining of cells and the deposition of stain in the cells into granules in *Limnobium* roots were determined by vital dye neutral red (0.01% aqueous solution) (Musienko et al., 1994). Exposure to solutions of nanoparticles and metal ions was 24 hours, and to dye solutions - 1 hour. All slides were viewed with light microscope MBI-15. Statistical analysis of the data was performed by analysis of variance.

## RESULTS AND DISCUSSION

Since it is known that nanoparticles can overcome protective barriers of cells, primarily easily penetrate cell membranes and even some organelle membranes, it is necessary to carry out vital staining of tissues after exposure of plant in the test solutions of metal nanoparticles for estimation their special features and effects on cytophysiological processes. Due to the fact that the investigated pleustophyta (limnobium and duckweed) contact with aqueous solutions mostly through roots, roots was chosen as appropriate object for cytomorphological study. While using duckweed some technical difficulties occured due to the small size of plants, so the main investigation has been conducted on *Limnobium*.

In response to the action of nanoparticles of biogenic metals in root cells of *Limnobium* a set of changes that characterize the general non-specific reactions to the impact of metal nanoparticles was observed. Stained root cells of *Limnobium* by vital dye neutral red under the influence of metal nanoparticles in colloidal form differed from the control (Table 1). Namely, disturbance of sediments of vital dyes in granules, formation of non-specific to the cells cubic and needle crystals (collected in the stellate formation), diffuse staining of protoplasm, and in some cases (Zn, Ag, the composition Cu-Zn) staining of cell nucleus, features of different stages of plasmolysis were admitted. Furthermore, volume of chloroplasts in root cortex cells (exoderm) of *Limnobium* increased, that evidenced of disturbance of semi-permeability of cell membrane and chloroplast membranes and impaired ion balance of cell.

**Table 1: Effect of metal nanoparticles on the degree of staining of root cells of *Limnobium laevigatum* by vital dye neutral red.**

| Metal nanoparticles | Type, the degree of staining and physiological state of cells |
|---|---|
| Control | Separation of stain into granules, uniform distribution of granules in cell |
| Fe-Mn | Separation of stain into granules, uniform distribution of granules in cell, absence of plasmolysis |
| Mn | Separation of stain into granules, uniform distribution of granules in cell, absence of plasmolysis |
| Fe | Separation of stain into granules, uniform distribution of granules in cell, absence of plasmolysis |
| Cu-Zn | The diffuse staining of the cytoplasm, formation of not-typical needle crystals, increasing of chloroplasts volume, angular plasmolysis |
| Zn | The diffuse staining of the cytoplasm, formation of not-typical cubic crystals, increasing of chloroplasts volume, cells are highly plasmolysed |
| Cu | Separation of stain into granules, not uniform distribution of granules in cell, formation of not-typical needle crystals gathered in the stellate sets, cells are highly plasmolysed |
| Ag | The diffuse staining of the cytoplasm, increasing of chloroplasts volume, cells are highly plasmolysed |

Metal nanoparticles (Zn, Ag, composition Cu-Zn) deduced the cell within an optimal physiological state and changed sharply the picture of intracellular distribution of vital dye. Increasing the ability of adsorbing vital dye by nucleus and cytoplasm is observed, however on the other hand, reducing the intensity of granule formation is also noticed. Since it is known that in normal conditions cytoplasm as well as nucleus of living cells do not absorb any vital dyes, our results suggest a toxic effect of the studied metal nanoparticles on the roots of *Limnobium*. Similar results were obtained in studies of the influence of metals in ionic form for *in vivo* staining *Limnobium* roots (Table 2).

**Table 2: Effect of $Cu^{2+}$ and $Zn^{2+}$ ions on the degree of staining of root cells of *Limnobium laevigatum* by vital dye neutral red.**

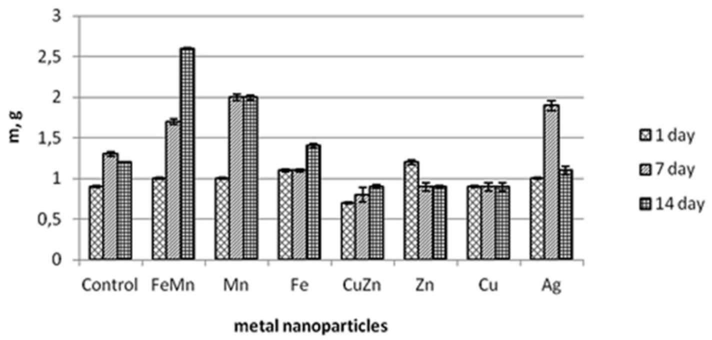
| Metal concentration, maximum allowable concentration (MAC) | Type, the degree of staining and physiological state of cells |
|---|---|
| Control | Separation of stain into granules, uniform distribution of granules in cell |
| $Cu^{2+}$, 100 | The diffuse staining of the cytoplasm, the formation of not-typical cubic crystals |
| $Cu^{2+}$, 10 | The diffuse staining of the cytoplasm, formation of not-typical needle crystals |
| $Cu^{2+}$, 1 | The diffuse staining of the cytoplasm, formation of not-typical needle crystals |
| $Zn^{2+}$, 100 | The diffuse staining of the cytoplasm, the formation of not-typical cubic crystals |
| $Zn^{2+}$, 10 | Separation of stain into granules, not uniform distribution of granules in cell, single cubic crystals |
| $Zn^{2+}$, 1 | Separation of stain into granules, uniform distribution of granules in cell |

The mechanisms of interaction of vital dyes and living cells are being studied intensively worldwide. Significant success of such researches is the identification of cytoplasmic organelles that are responsible for the intracellular accumulation of the vital dye in granules. It was demonstrated recently that the granules observed under the microscope, occurred to be lysosomes with accumulated stain. The universal function of the lysosomes in degradation of any unwanted substances invaded into the cell was also determined.

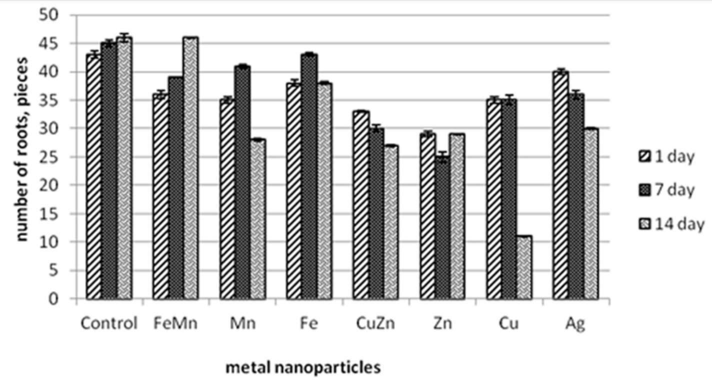
Obtained results according to the methods of bioassay based on growth reaction also point to the ambiguous impact of biogenic metal nanoparticles on aquatic plants. Various metals and their binary compositions revealed both stimulating and highly inhibitory effects (Fig. 1).

On the 7th day of the experiment morphological parameters of *Limnobium* under the influence of biogenic metal nanoparticles Fe and Mn were not different from control option. All the plants were normal size, bright green, with shiny glossy surface of the leaves, well-developed root system of a pale green color. Mass of plants of control and all variants of treatment by nanoparticles of biogenic metals increased. Number of roots and leaves significantly increased, the total number of individual plants also raised. New daughter plants developed normally and had bright green leaves. Thus, obtained results allow us to conclude the absence of any adverse effect of biogenic metal nanoparticles of Fe and Mn on the 7th day of exposure.

A




B




C

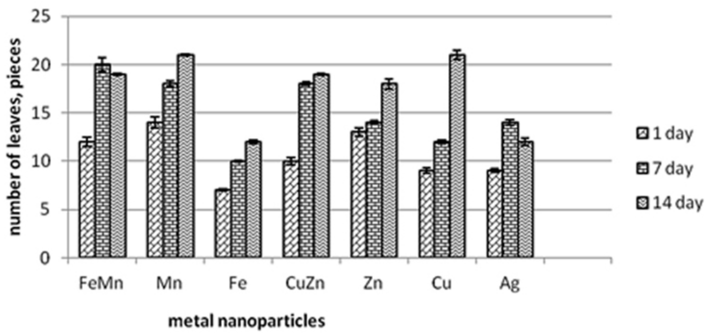



**Fig. 1. Influence of nanoparticles of biogenic metals on growth reactions of *Limnobium laevigatum.* A – plants weight; B – number of leaves; C – number of roots.**

Under the influence of the Fe-Mn composition adult plant developed normally, daughter plants retained bright green color, roots, visually, became more subtle at the same time their number increased, as well as increased mass of plants. Effect of Mn on roots did not result in any change: roots retained normal, long and branched enough. Older leaves remained intensively green; an increase number of young leaves on the initial plants, daughter plants formation, biomass growth and multiplication of leaves and roots were observed. Under the influence of Fe leaves were intensely green; roots remained in a normal state and did not differ from the control. Positive tendency in terms of growth was admitted: weight of the initial plants increased, the number of leaves and roots, as well as the number of plants outlets, on account on new subsidiaries individuals increased. Fe, Mn nanoparticles and Fe-Mn composition stimulated plant growth.

Leaves of initial plants discolored first, then majority died off, and the rest were in an extremely depressed state under the influence of Zn. Roots changed color to brown, decay of the roots from the tip to the base was observed. Nanoparticles of Cu caused partial death of leaves and roots of the initial plants and discoloration of leaves of subsidiary plants. Composition Cu-Zn also did not effected favorably on plants. New roots stunted and old ones died out by 9% and 18% on the $7^{th}$ and $14^{th}$ day of exposure respectively. Plant leaves have changed color to a light green, daughter plants turned yellow immediately after formation.

Leaves of the initial plants were light green, and the newly formed daughter plants discolored, their roots gradually died out under the influence of Ag. Plants weight initially almost doubled by $7^{th}$ day of exposure, on the $14^{th}$ day decreased to the original value. The number of roots did not increase, but rather gradually decreased: on the $7^{th}$ day by 10%, and on the $14^{th}$ day - by 25%, compared with control (Fig. 1).

Thus, on the $14^{th}$ day of the experiment morphological parameters of *Limnobium* in all researched options changed significantly, both in the presence of individual metal nanoparticles as well as their binary compositions.

Our results showed favorable impact of Mn nanoparticles and Mn-Fe composition on growth reaction of leaves, roots and subsidiary plant of *Limnobium*. Number of plants doubled in both cases, treatment with Mn and Mn-Fe composition resulted in 2-fold and 2,6-fold rising of plants weight during 14 days of exposure respectively, at the same time these indexes increased only by 33% in control plants comparing with the initial state. The number of leaves and roots of plants enhanced: under the influence of Mn by 50% and 37%, and under the influence of the composition Mn-Fe by 58% and 27% respectively. At the same time inhibitory effect of Zn nanoparticles for biomass growth and the formation of subsidiaries of individuals starting from the $7^{th}$ day was admitted. Then on the $14^{th}$ day leaves or even entire adult individuals started to die out, their growth inhibited and morphological changes such as yellowing and drying of the leaves, and the subsequent decay of dying roots were observed. Although under the influence of Zn in the composition with Cu such a negative effect was leveled: number of new subsidiary individuals increased compared with control. Nanoparticles of Ag significantly stimulated the plants growth on 7th day of experiment: the mass of plants increased nearly 2-fold compared with the initial, and the number of leaves increased almost in 1.5 times. But at the same time the number of roots reduced by 10% compared with baseline values. The negative impact of Ag on all researched parameters increased at 14th day of exposure.

A

B

C

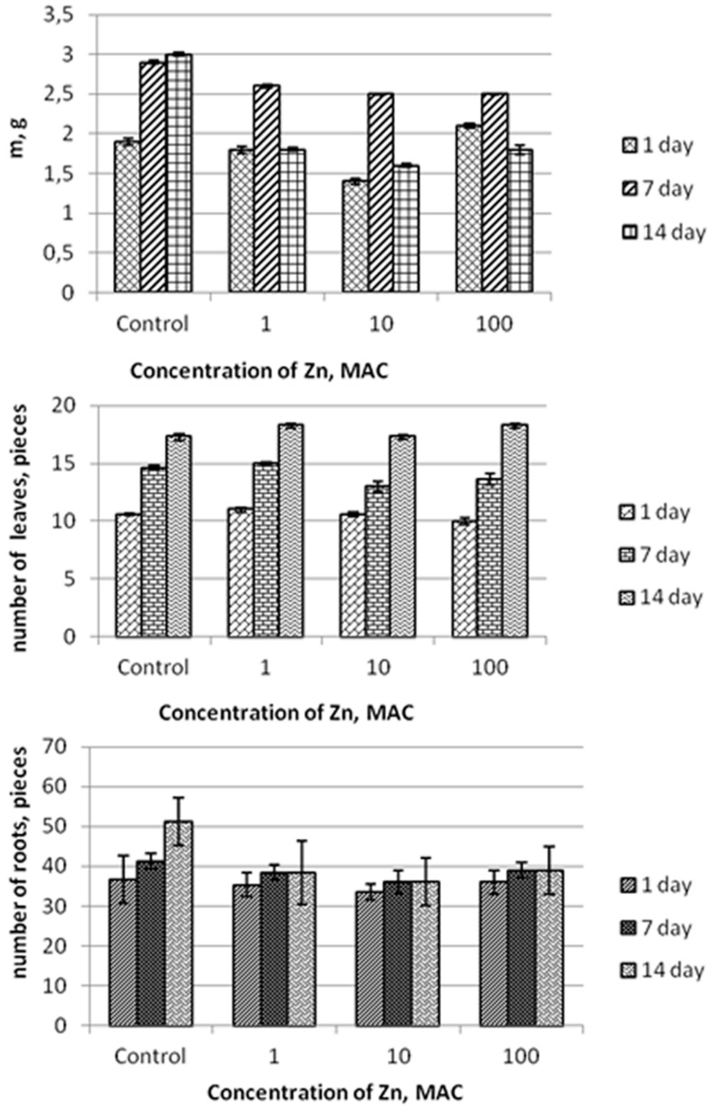



**Figure 2. Influence of Zn ions on growth of *Limnobium laevigatum*. A – plant weight; B – number of leaves; C – number of roots.**

Thus, in our studies it was found that nanoparticles of biogenic metals such as Zn, Cu, and composition Zn-Cu and Ag, instead of the expected stimulating effect on the contrary caused adverse changes in growth of *Limnobium*. Unlike nanoparticles of Fe, Mn and their composition stimulated plant growth during the experiment.

The impact of Zn, which revealed to be toxic in the form of colloidal nanoparticles for growth of *Limnobium,* has also been studied in the ionic form of the metal in laboratory experiment (Fig. 2).

On the 24 hour of the experiment significant changes in the morphological indicators were not identified. Plants remained green and continued their intensive developing at all tested concentrations of the metal.

On the 7$^{th}$ day some positive changes in the morphological indices were admitted: the mass of plants both in the control and in all researched concentrations of Zn ions increased, the number of roots, leaves and individual plants due to the newly-formed subsidiary individual's outlets, also raised. Plants were intensely green, developed normally, so the negative impact of metal was not found.

On the 14$^{th}$ day of the experiment significant morphological changes, manifested by both visual and quantitative effects were marked. In the control plants leaves remained green, alive, normal size, while the old ones gradually died out. The number of leaves and roots increased over the 7$^{th}$ day. At a concentration of Zn 1 MAC older plants appeared normal, but the young leaves were brighter than the old ones. This is the evidence of the negative impact of metal ions on *Limnobium* already at the lowest tested concentration. At a concentration of 10 MAC leaves of experimental samples differed in color from the control option, but a few remained green, all other kept yellowing or became discolored, large necrotic spots on the leaves appeared, the newly formed leaves were smaller compared to the control, and there was intensive dying of roots. Negative effect of Zn ion concentration of 100 MAC even more amplified. Another representative of the same ecogroupe of pleustophyta, namely *Lemna minor,* was used for determination the toxicity of zinc and copper in the ionic form that was showed above for *Limnobium*.

It has been found empirically that the Cu concentration in the investigated range from 1 to 100 MACs did not have a negative impact on growth of *Lemna minor*, while the corresponding Zn concentration had a deleterious effect on the same parameters (Fig. 3).

Even at concentrations of 1 and 10 MAC initial plants changed their color from bright green to light green, while maintaining the normal size of individuals, leaf color of subsidiary plants was not changed, however, the number of leaves significantly decreased compared to control. Also, we noticed the extinction of initial plants. At a concentration of 100 MAC coloring of the initial plants changed to light yellow, and the newly forming subsidiary plants first became colorless, and eventually died out.

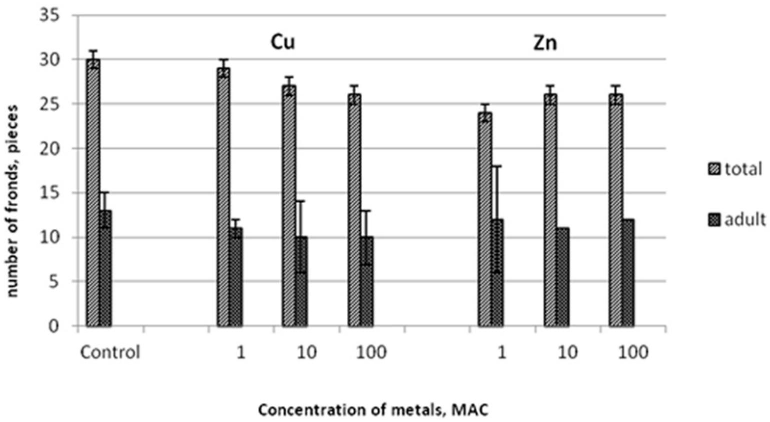



**Figure 3. Influence of Cu and Zn ions on growth of *Lemna minor* on 7th day of exposure.**

## CONCLUSION

Results of cytophysiological and morphological research as well as studying of growth parameters were of the same type. Namely, the effect of nanoparticles of metals Mn, Fe, and their binary composition Fe-Mn were favorable for the aquatic plants, on the other hand, Zn, Cu, Ag nanoparticles, and binary composition Cu-Zn showed deleterious effects. Results of experiments with *Lemna* were compatible with the results obtained on *Limnobium*, showing similar growth response of plants belonging to one ecological group to the damaging effects of metals Zn and Cu, without any decisive importance of metal form – nanoparticle or ionic. As well as in ionic form, Zn and Cu in the form of nanoparticles showed high toxicity, which indicates the potential danger of nanomaterials based on them to aquatic organisms.

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