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QUANTITATIVE INDICATORS OF COPPER-RESISTANT MICROORGANISMS DISTRIBUTION IN NATURAL ECOSYSTEMS

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Copper is a highly toxic metal common in both natural and man-made ecosystems. The goal of the work was to determine the level of resistance of microorganisms of natural ecosystems to cationic form and organometallic complex of Cu^{2+}. Microorganisms of 9 natural ecosystems of five geographic zones (the Antarctic, the Arctic, the Dead Sea (Israel), middle latitude (Ukraine) and the equatorial zone of South America (Ecuador) were investigated. Resistance of microorganisms was determined by cultivation in the medium with concentration gradient of Cu^{2+}. The amount of Cu^{2+}-resistant microorganisms in natural ecosystems was determined by colony counting on nutrient agar with Cu^{2+} citrate and Cu^{2+} cation. The Cu(II) concentration in soil and clay samples was analyzed by atomic absorption spectroscopy method. We have confirmed the hypothesis that microorganisms resistant to toxic Cu^{2+} compounds in high concentrations exist in any natural ecosystem. The resistance to Cu^{2+} cation was 8–31 and 14–140 times less than to Cu^{2+} citrate in nutrient and mineral agar media respectively. The amount of Cu^{2+}-resistant microorganisms in natural ecosystems reached hundreds and thousands at the presence of 175–15 500 ppm Cu^{2+}. Thus, the soils, clays and sands of natural ecosystems are a "genetic resource" of copper-resistant microorganisms that are promising for development of novel biotechnology of purification of copper-containing wastewater and soil bioremediation.

Key words: copper pollution, copper-resistant microorganisms, natural ecosystems, environmental biotechnologies.

Copper is a necessary trace element for microorganisms, but in high concentrations it is a toxic metal [1]. The origin of metal ores on the Earth is associated with the activity of microorganisms. The role of bacteria in the formation of the so-called sedimentary copper ores [2] and the phenomenon of copper resistance [3] were described in the middle of XX century. Copper compounds are common in both natural and man-made ecosystems [4]. Deposits and mines, industrial enterprises as well as agricultural activity are the main sources of copper pollution [5, 6]. The concentration of copper reaches tens of grams per 1 kg of soil in places of contamination [7]. The investigation of copper-resistant microorganisms (CRM) has both theoretical and applied significance. These microorganisms are able to extract copper(II) compounds from solutions by the following mechanisms, such as accumulation in cells, precipitation of Cu^{2+} in forms of $Cu(OH)_2\downarrow$, $CuCO_3\downarrow$ and also reduction to insoluble compound $Cu_2O\downarrow$ [8]. We assume that these properties were inherent in microorganisms at ancient time during the formation of copper minerals. Now they are promising for the development of biotechnologies for purification of copper industrial sewage and contaminated ecosystems.

Thus, CRM are important in the biogeochemical cycles of copper compounds transformation in natural ecosystems. Based on this, on our opinion, they must be present in both natural and man-made ecosystems in large amounts. The efficiency of transformation of copper compounds depends on both its concentration and the amount of CRM. Therefore, the quantitative determination of CRM in natural ecosystems complements the theoretical knowledge about their contribution to the functioning of biogeochemical cycles. Natural ecosystems are separate from man-made pollution and contain trace concentrations of toxic heavy metals, including copper [9]. However, we suppose that natural ecosystems contain copper-resistant microorganisms, because adaptation to high concentration of heavy metals and interaction with them are ancient properties of microorganisms.

In this regard, the purpose of our work was to estimate the amount of CRM in natural ecosystems of different geographical zones of the globe. In general, to prove CRM widespread distribution in natural ecosystems.

Materials and Methods

Microorganisms have been isolated from 9 natural ecosystems of five geographic zones. These zones included the Antarctic, the Arctic, the Dead Sea (Israel), middle latitude (Ukraine) and the equatorial zone of South America (Ecuador). The samples were collected in sterile zip bags and transported after being placed in containers with salt and ice (the ratio of 1/1) to stabilize a low temperature (– 18...– 9 °C) during samples transportation. After transportation to the laboratory, the samples were stored at −20 °C [10, 11]. This technique minimized the decrease of viable microorganisms.

Microorganisms resistant to Cu(II) were detected by their ability to grow on the agar nutrient medium (NA, HiMedia Laboratories Pvt. Ltd., India) and agar mineral medium (MM) with Cu(II) compounds.

Two modifications of copper(II) compounds were used in the experiments to compare the resistance of microorganisms to cationic form and to organometallic complex of Cu(II). The first modification was the soluble cation Cu^{2+}. It was added into the nutrient medium in the form of a copper sulfate salt $CuSO_4$.

The second modification was the soluble organometallic complex of Cu^{2+} with three substituted sodium citrate — $[Cu^{2+} \times Na_3cit]$.

The solubility of $[Cu^{2+} \times Na_3cit]$ in the nutrient agar (NA) was 15 500 ppm, and non-chelated form of Cu^{2+} ($CuSO_4$) — 1 100 ppm. The solubility of Cu^{2+} in the mineral medium depended on the sulfate content. The composition of the mineral medium included (g/L): NH_4Cl — 1.0; Na_2SO_4 — 0.5; K_2HPO_4 — 0.5; $C_6H_{12}O_6$ — 5.0; agar — 20. The phosphate content was reduced because at its higher concentration, Cu^{2+} precipitated.

The solution of 40 000 ppm Cu^{2+} was used as the stock. It was prepared by $CuSO_4 \cdot 5H_2O$ dissolving in distilled water in a volumetric flask. To obtain copper citrate, after complete dissolution of the sulfate, citrate crystals were added to the copper solution.

The 25.0 ml of agar medium with Cu^{2+} in citrate or sulfate forms and copper-free control medium were added to the plates. The plates kept for 2 days at 25 °C to check the sterility and drying of agar surface. For inoculation of microorganisms, ten-fold dilutions of the sample were prepared, of which 0.2 ml of a suspension of microorganisms ($n \times 10^{-2}$) was inputted on the agar surface and rubbed with a spatula. Copper-free medium was used as a control. Microorganisms were cultured at 30 °C for 10 days. The amount of microorganisms was calculated on their content in 1 g of completely dry soil [12].

According to our definition, homeostasis is the stable existence of microorganisms under the action of extreme factors.

A maximum permissible concentration (MPC), the amount of copper-resistant microorganisms (CFU/g), as well as concentration coefficient of resistance (K_R) served as the representative quantitative indicators of homeostasis. They characterize the adaptation potential of microbiomes to the action of toxic Cu(II).

A maximum permissible concentration (MPC) corresponds to a maximum concentration at which the growth of microorganisms is still possible.

A colony-forming unit (CFU) is a unit used in microbiology to estimate the amount of viable bacteria or fungal cells in a sample [12].

The concentration coefficient of resistance (K_R) characterizes how much time the MPC of toxic metals for microorganisms exceeds their content in the habitat. The coefficient K_R was determined by the formula:

$$K_R = MPC:C$$

where MPC — a maximum permissible concentration of Cu(II); C — concentration of soluble copper in research sample.

A reference content of soluble copper in soils (3 mg/kg) was used for K_R calculation for microorganisms of “Atlantida” karst cave [18, 19].

Resistance of microorganisms was determined by cultivation in the medium with concentration gradient of Cu^{2+}. The concentration gradient differed during cultivation of different microbiomes. For example, for cultivation of “Optymistychna” cave microorganisms, gradient ranged from 200 to 4 000 ppm Cu^{2+} with a step — 200 ppm. The Cu^{2+} concentration in the nutrient medium was increased with different steps. The minimum step was 1 ppm, the maximum — 500 ppm. The amount of Cu^{2+}-resistant microorganisms in natural ecosystems was determined by colony counting on nutrient agar with $[Cu^{2+} \times Na_3cit]$ and Cu^{2+} cation. The Cu(II) concentration in soil samples of different geographical zones was analyzed by atomic absorption spectroscopy method [13]. Accumulation of Cu(II) in colonies was confirmed by H_2S test [14]. The essence of the method is a visualization of Cu^{2+} that accumulated in colony by H_2S. The presence of Cu^{2+} in the colonies was evidenced by the appearance of dark brown or black Cu(II) sulfide — $CuS\downarrow$ after treatment by H_2S. The reduction of Cu(II) was shown by appearing of brown color due to the formation of insoluble $Cu_2O\downarrow$ in colonies [8].

Results and Discussion

All investigated ecosystems were differed by complex of extreme factors (high and low temperatures, high salinity, UV radiation, etc.). Ecosystems of the Arctic and Antarctic are characterized by the effects of very low temperatures, ranging from — 40 ... + 6 °C. Ecosystem of the Dead Sea are characterized by exposure to excessive UV-radiation, as well as high temperatures — up to 40–50 °C. Ecuadorian ecosystem has also been affected by high temperatures up to 50 °C. Despite the temperature difference, the intensive effect of UV-radiation is common for Arctic and Antarctic, as well as Ecuador and the Dead Sea ecosystems. Unlike above named extreme ecosystems, cave ecosystems are free from the effects of extreme factors. For example, karst caves ecosystems “Optymistychna” and “Atlantida” were isolated from any man-made contaminants and characterized by the influence of temperate low temperatures (12–14 °C) as well as complete absence of toxic metals (Cu^{2+} in particular) and UV radiation. Natural ecosystems were shown to contain Cu(II) in trace concentrations ranging from 7.6 to 27.2 ppm (mg/kg) of sample (Table 1). Thus, the lowest concentration of copper was observed in the sample from the Dead Sea, the highest — in the ecosystem of the “Optymistychna” karst cave (Table 1).

We have confirmed the hypothesis that microorganisms resistant to toxic Cu(II) compounds in high concentrations exist in any natural ecosystem. Thus, the amount of resistant to Cu^{2+} (1000 ppm, in the citrate form) microorganisms ranged from 2.3×10^3 (Israel, the Dead Sea) to 1.8×10^6 CFU/g (Ukraine, Kyiv region) (Fig. 1).

For the first time, we discovered that super-high copper-resistant microorganisms are widespread in natural ecosystems. Microorganisms are resistant to high concentration of Cu^{2+} (up to 15 500 ppm) were present in all investigated ecosystems (Table 1). At first consideration, the survival of microorganisms at such high concentrations of copper contradicts the generally accepted notion about bactericidal properties of Cu^{2+} in the concentration range 20–100 ppm [15]. However, the amount of microorganisms decreased significantly with the increase of copper concentration confirming the toxicity of copper to microorganisms.

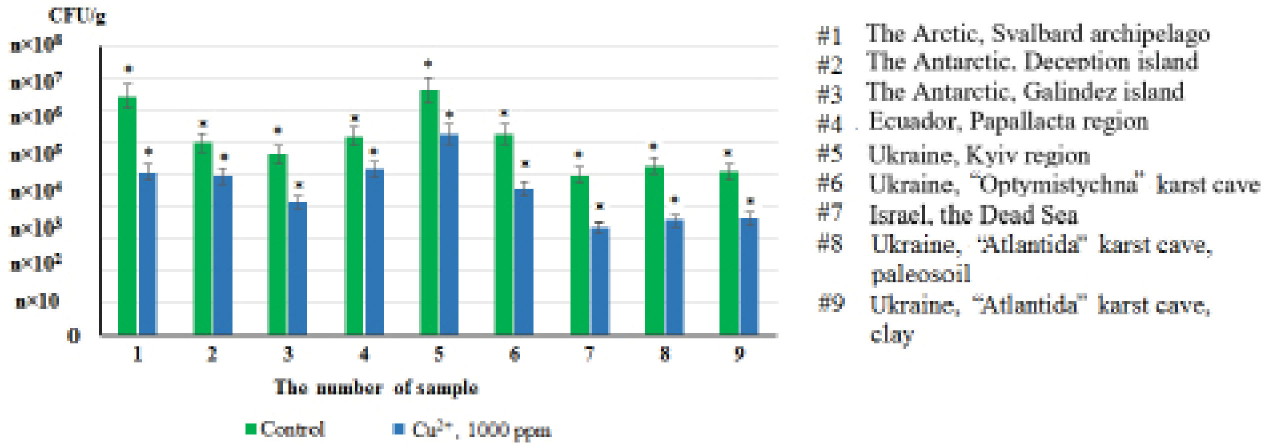

Fig. 1. **High resistance of the microorganisms of 9 natural ecosystems to toxic Cu^{2+} citrate**

Table 1. **Quantitative characteristic of copper-resistant microorganisms in natural ecosystems**

N of the sample	Ecosystem, Location of sampling	Isolation source	The copper concentration in the investigated samples, ppm (mg/kg) of sample	The amount of CRM* on Nutrient Agar with 1000 ppm Cu^{2+}, CFU/g	The maximum permissible concentration (MPC) on Nutrient Agar with Cu^{2+}, ppm	The amount of CRM* on Nutrient Agar with MPC of Cu^{2+}, CFU/g
1	The Arctic, Svalbard archipelago	Soil	22.6	$1.12\cdot10^5$	3 800	$1.5\cdot10^3$
2	The Antarctic, Deception island	Soil	13.5	$8.9\cdot10^4$	15 500	$5.0\cdot10^2$
3	The Antarctic, Galindez island	Soil	23.2	$1.33\cdot10^4$	8 500	$1.1\cdot10^2$
4	Ecuador, Papallacta region	Soil	23.6	$1.4\cdot10^5$	4 000	$1.0\cdot10^3$
5	Ukraine, Kyiv region	Soil	8.3	$1.8\cdot10^6$	10 500	$7.6\cdot10^2$
6	Ukraine, "Optymistychna" karst cave	Clay	27.2	$3.5\cdot10^4$	4 000	$1.28\cdot10^3$
7	Israel, the Dead Sea	Sand	7.6	$2.28\cdot10^3$	600	$5.07\cdot10^2$
8	Ukraine, "Atlantida" karst cave	The paleo soil	–	$3.7\cdot10^3$	2 400	$1.85\cdot10^3$
9	Ukraine, "Atlantida" karst cave	Clay	–	$4.25\cdot10^3$	2 000	$7.0\cdot10^3$

Note: *CRM — copper-resistant microorganisms.

The issue about level of the toxicity of different copper compounds to microorganisms is very important [1]. Depending on the modification of copper, the level of its toxicity varies. For example, insoluble copper compounds ($Cu(OH)_2\downarrow$, $CuCO_3\downarrow$ etc.) are low-toxic or completely non-toxic to microorganisms. Soluble compounds of copper(II) are toxic and inhibit the growth of microorganisms [16], because the redox systems formed by them are characterized by high values of redox potential (Eh) [8]. On the example of the microbiome of chernozem soil, we have previously shown that the toxicity of $CuSO_4$ exceeds significantly the toxicity of Cu(II) in complex with citrate. It was found that chelation by citrate increases the resistance of microorganisms to Cu^{2+} in 20 times [17]. It is obvious that the high toxicity of $CuSO_4$ is determined not only by toxic properties of Cu^{2+}, but also by low pH values (3.2–3.5) and consequently high values of redox potential up to +580 mV. The chelation of Cu^{2+} by three substituted sodium citrate ($Na_3C_6H_5O_7$) stabilized the obtained complex in wide range of pH (pH = 0.0–9.0). That is why the neutralization of acid solution by alkaline sodium citrate inevitably leads to decrease the redox potential to +300 mV. It allows to neutralize the solution to 6.5–7.0, which are optimal for the growth of microorganisms.

Herein, the MPC of the studied microbiomes to Cu^{2+} in complex with citrate in nutrient agar and mineral media was high and ranged from 175 to 15 500 ppm Cu^{2+}. For example, the most resistant were the Antarctic microorganisms isolated from biogenesis of Deception Island (sample #2, Table 1, Fig. 2). On the contrary it was shown that resistance of microorganisms to non-chelated Cu^{2+} cation was in 31 times lower, only 500 ppm Cu^{2+} (Fig. 2).

Resistance of this microorganisms to Cu^{2+} in NA and MM media did not differ. Thus, the MPC in both case was 500 ppm Cu^{2+}. However, the MPC of Cu^{2+} in citrate form in mineral medium was in 11 times lower than in nutrient agar (Fig. 2; #2 sample). Really, the MPC of Cu^{2+} in citrate form was 15 500 ppm, in form of Cu^{2+} cation was 1400 ppm (15 500:1400 = 11.07).

This difference may be caused by the presence in the NA of additional chelators — amino– and organic acids. They caused a strong stabilization of pH and Eh parameters of medium at optimal level and as a result increase the microorganisms resistance to Cu^{2+}.

The microorganisms from the "Atlantida" karst cave (Fig. 2, #8 sample) had the lowest resistance to Cu^{2+} cation. The resistance of this microorganisms in the mineral medium was critically low. Thus, only 2 ppm of Cu^{2+} in

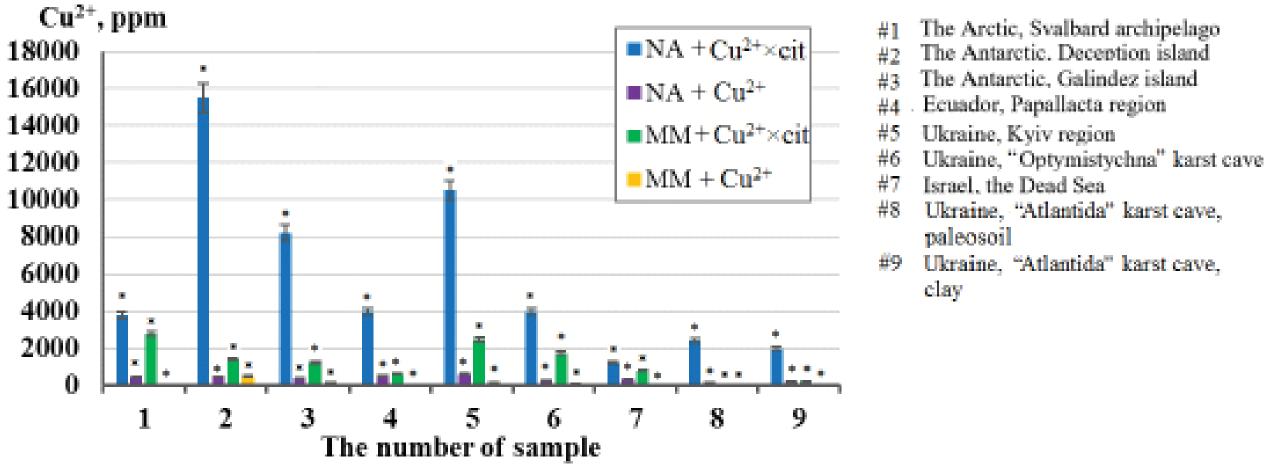

Fig. 2. **Comparison of MPC of microorganisms isolated from microbiocenoses of differ-ent geographical zones of the globe to soluble Cu^{2+} compounds ($P \le 0.05$)**

cation form led to a catastrophic decrease of the amount of living microorganisms. At the same time, the maximum permissible concentration of Cu^{2+} in sulfate form in NA medium was in 87.5 times higher (175:2 = 87.5) (Fig. 2, Table 2). Obviously, another compounds with properties of chelators are also present in the rich NA medium. They responsible for chelation of toxic Cu^{2+} cation and as a result play a crucial role in the microbial resistance.

Despite the high toxicity of copper compounds, the amount of living microbial cells in natural ecosystems reached hundreds and thousands at the presence of 175–15 500 ppm Cu^{2+}. These copper-resistant microorganisms can be divided into three groups: low-resistant (20–175 ppm Cu^{2+}), medium-resistant (175–1000 ppm Cu^{2+}) and highly resistant (1000 ppm to 15 500 ppm Cu^{2+}). Notwithstanding the fact that some are more or less resistant, in all ecosystems there are microorganisms that are resistant to concentrations that considered as bactericidial nowadays. Thus, the microorganisms of Antarctic (Deseption island) was highly-resistant to Cu^{2+} (MPC = 15 500 ppm). The amount of CRM was 5.0×10^2 CFU/g at this super-high concentration.

At first consideration, the existence of super-high copper-resistant microorganisms in the natural ecosystems contradicts the generally accepted idea of the bactericidal properties of Cu^{2+} in the concentration range of 20–100 ppm [15, 16]. However, our results show that the increase of Cu^{2+} concentration leads to the significant decrease of the amount of living microorganisms (Fig. 3–6). Thus, we experimentally confirmed the general biological regularity of the toxic effect of copper on microorganisms. Fundamentally new is the fact that even at the MPC of Cu^{2+}, the amount of microorganisms was hundreds and thousands of viable cells (Table 1). This fact can be explained by the wide variety of microbial communities in the natural ecosystems and their genetically determined ability to adapt to extreme factors.

In the mineral medium, the resistance of microorganisms to Cu^{2+}, citrate was slightly lower than in NA. Under such conditions, the microorganisms from Arctic ecosystem was the most resistant to Cu^{2+}, (#1 sample, Tables 1, 2, Fig. 4). The MPC of Cu^{2+}, was 2800 ppm. At such conditions, the number of resistant microorganisms was high — 1.5×10^3 CFU/g.

Unlike the Arctic microorganisms, the microorganisms of paleosoil and clay ("Atlantida" cave) were not highly resistant both to Cu^{2+} citrate and Cu^{2+} cation. The amount of living cells decreased catastrophically with a slight increase of the concentration of Cu^{2+} citrate. The amount of living cells decreased from 8.4×0^3 to 1.9×10^3 CFU/g (paleosoil) and 6.4×10^2 CFU/g (clay) at 50 ppm and 200 ppm Cu^{2+} citrate respectively (Fig. 5). Such low resistance may be caused by the low concentration of microorganisms in the microbiocenoses of clay and soil, which are able to grow at the presence of carbohydrates (glucose) as sources of carbon and energy. The results show that the microorganisms that grow well on protein-rich nutrient media were dominated in the investigated cave ecosystems.

The resistance of microorganisms to Cu^{2+} cation was significantly lower than to the Cu^{2+} citrate (Table 2; Fig. 5).

Table 2. **Resistance of microorganisms isolated of natural ecosystems to toxic Cu^{2+} compounds in different modifications and media**

Characterization of samples	**[Cu^{2+}×cit] + NA, MPC, ppm**	**Cu^{2+}+ NA, MPC, ppm**	**[Cu^{2+}×cit] + MM, MPC, ppm**	**Cu^{2+}+ MM, MPC, ppm**
#1, The Arctic, Svalbard archipelago	3 800	450	2 800	20
#2, The Antarctic, Deception island	15 500	500	1 400	500
#3, The Antarctic, Galindez island	8 500	400	1 200	150
#4, Ecuador, Papallacta region	4 000	500	600	10
#5, Ukraine, Kyiv region	10 500	600	2 600	175
#6, Ukraine, “Optymistychna” karst cave	4 000	250	1 800	100
#7, Israel, the Dead Sea	1 200	350	800	25
#8, Ukraine, “Atlantida” karst cave (paleosoil)	2 400	175	1	2
#9, Ukraine, “Atlantida” karst cave (clay)	2 000	200	200	3

Note: NA — nutrient agar; MM — mineral medium; MPC — maximum permissible concentration.

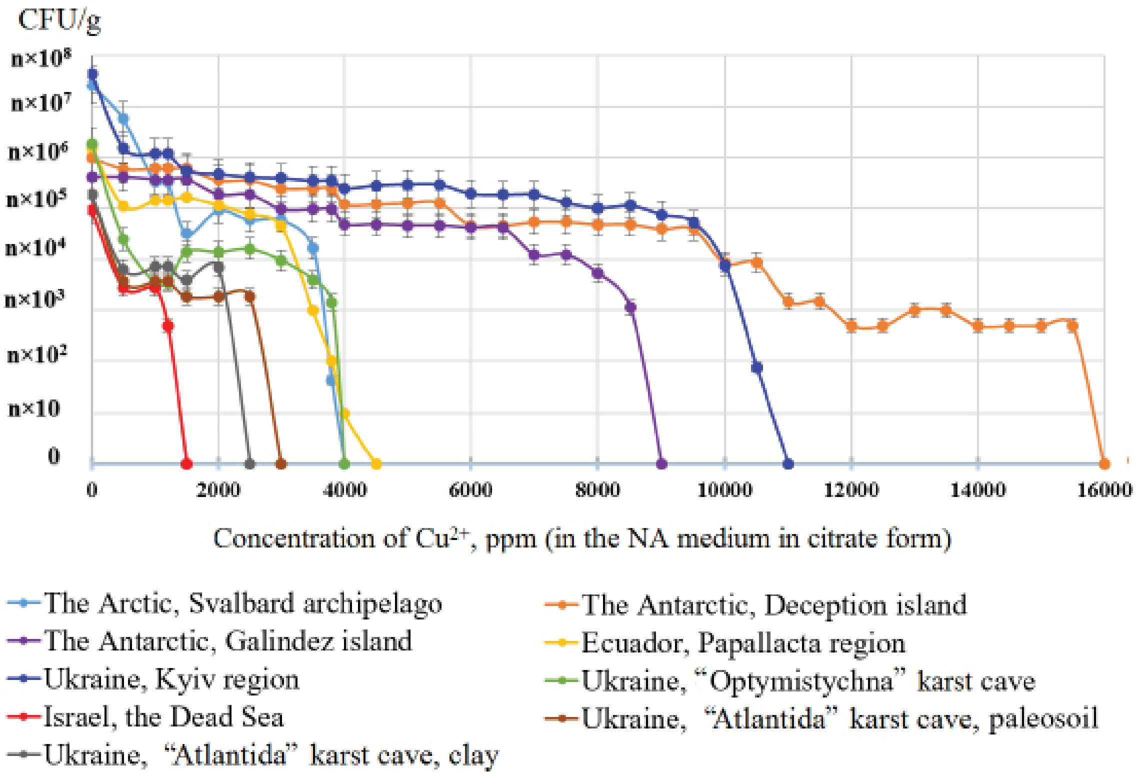

Fig. 3. **Cu^{2+}-resistance of microorganisms isolated from natural ecosystems ($P \le 0.05$)**

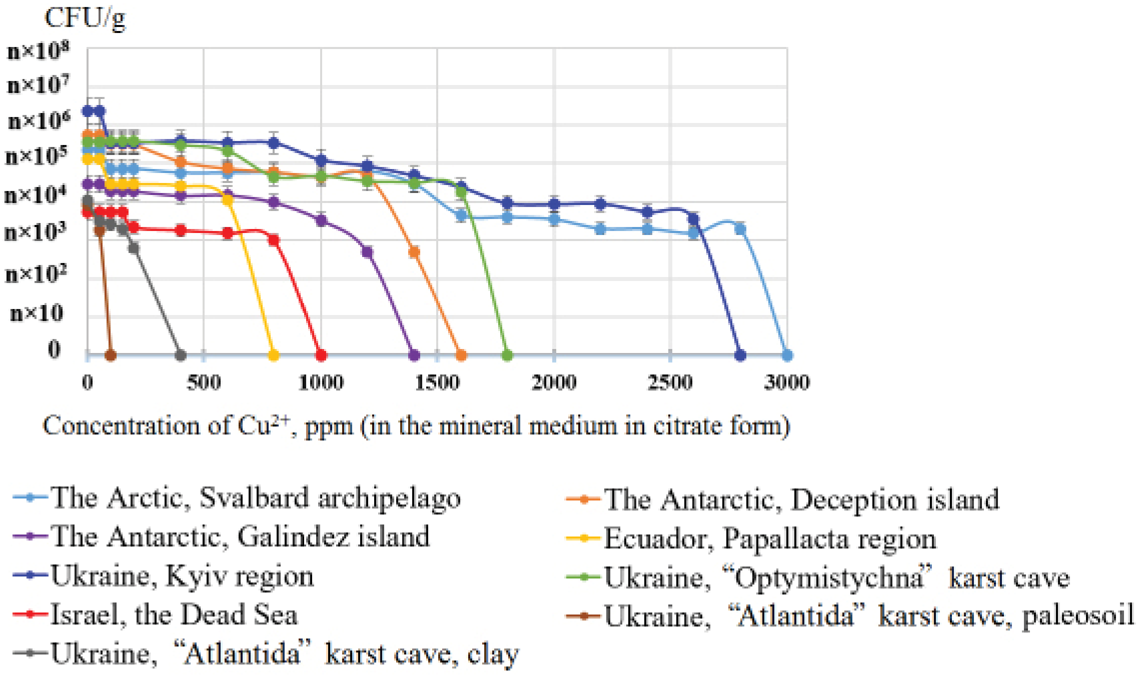

Fig. 4. **Resistance of microorganisms of natural ecosystems to Cu^{2+} citrate ($P \leq 0.05$)**

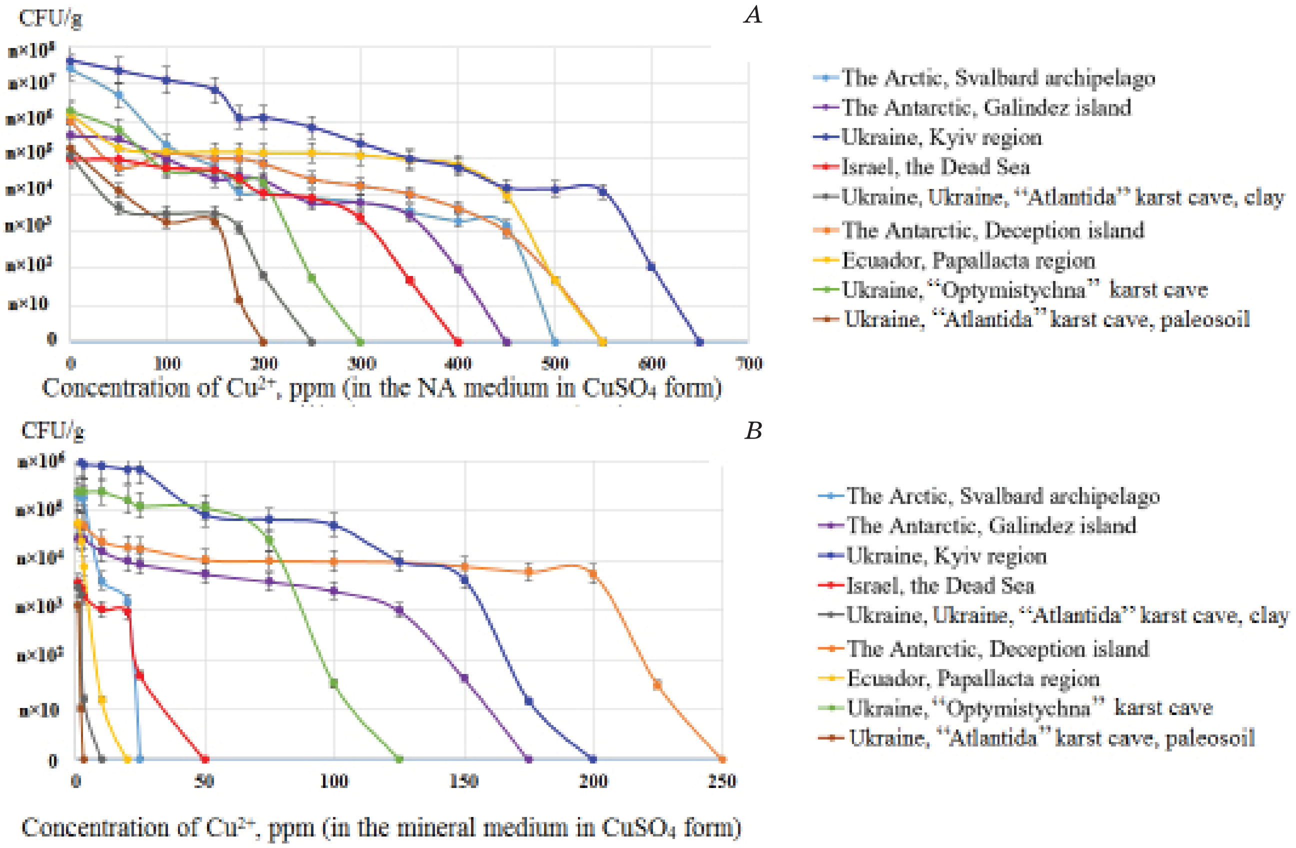

Fig. 5. **Resistance of natural ecosystems microorganisms to toxic Cu^{2+} in citrate form:**
A — in the NA medium; *B* — in the MM medium) ($P \leq 0.05$)

The resistance to Cu^{2+} cation was 8–31 and 14–140 times less than to Cu^{2+} citrate in NA and MM media respectively. The microbiomes were not highly resistant to Cu^{2+} cation in the MM medium. There are no additional chelators and stabilizers of pH and redox potential. This caused the low resistance of Cu^{2+}, which directly depends on these thermodynamic parameters. Thus, the MPC Cu^{2+} cation ranged from 175 ppm (Ukraine, «Atlantida» carst cave) to 600 ppm (Ukraine, Kyiv region) in a rich NA medium. The amount of living microbial cells under the action of the MPC of the non-chelated form of Cu^{2+} ranged from 4.4×10^2 CFU/g (the Antarctic, Galindez island, mineral medium, Fig. 6, *B*) to 1.1×10^4 CFU/g (Ukraine, Kyiv region, NA medium, Fig. 6, *A*).

We confirmed the evidence of two types of microorganisms response in the concentration gradient of Cu^{2+} compounds. The first type of response (correlative) is characterized by the correlative and catastrophic decrease of the amount of living microbial cells in gradient of copper. For example, the "Atlantida" cave microorganisms were resistant only to 2 ppm of Cu^{2+} (Fig. 6, *B*). Thus, in the response of the first type there were the following changes in the amount of living microbial cells. In the control variant without Cu^{2+}, the amount of living microbial cells was 1.1×10^4 CFU/g. The amount of microorganisms decreased in 8.6 times to 1.28×10^3 CFU/g with increasing Cu^{2+} concentration to 1 ppm Cu^{2+}, and to 1.1×10^3 CFU/g at 2 ppm Cu^{2+}. The microorganisms did not survive with the subsequent increase of Cu^{2+} concentration. The second type of response is non-correlative, in which case a significant increase of the copper concentration does not lead to a catastrophic decrease of living cells of microorganisms (The Arctic, Svalbard archipelago and Deception island microorganisms, as well as microorganisms of Ukraine, Kyiv region). With this type of response, the concentration of microorganisms remained at the same level with increasing concentration of Cu^{2+} from 6 000 to 9 000 ppm (Fig. 3, the Antarctic, Deception Island).

Thus, we have shown that in natural ecosystems there are microorganisms that are resistant to very high concentrations of toxic copper compounds and significantly exceed

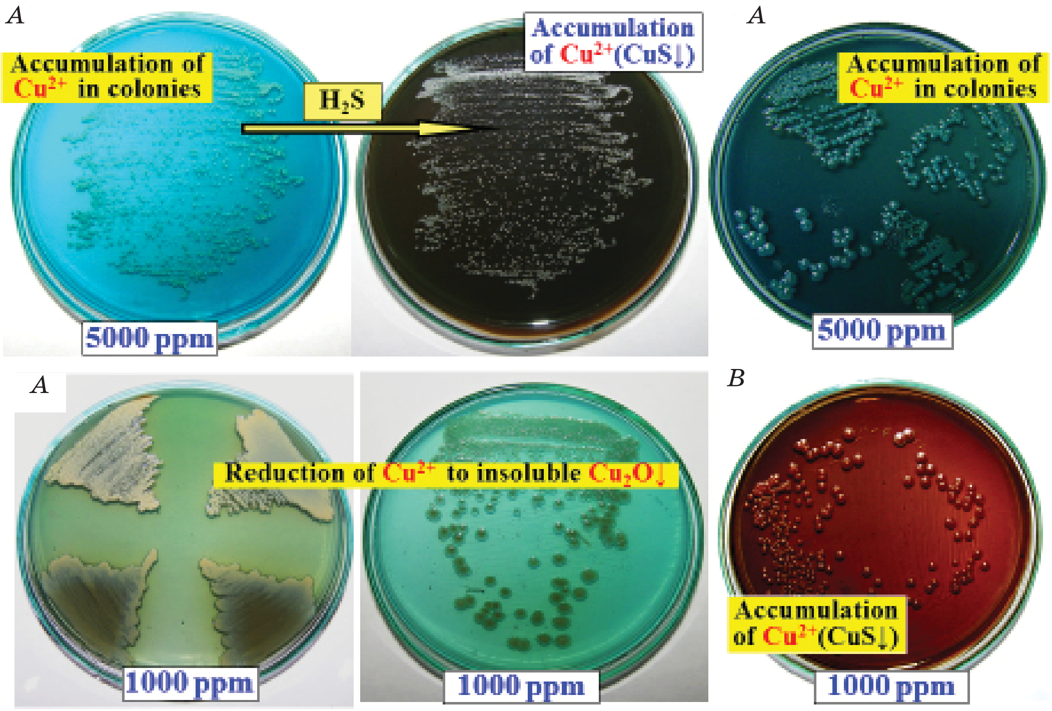

Fig. 6. **The growth and interaction of isolated industrially promising strains of copper-resistant microorganisms with toxic Cu^{2+}:**
A — pure culture iso-lated from Ukrainian soil (#5 sample, Kyiv region);
B — pure culture isolated from Antarctic soil (#2 sample, Deseption Island)

Table 3. **Values of concentration resistance coefficients under different cultivation conditions**

No of sample	Characterization of samples	K_R in the NA with $[Cu^{2+} \times Na_3cit]$	K_R in the NA with Cu^{2+} cation	K_R in the MM with $[Cu^{2+} \times Na_3cit]$	K_R in the MM with Cu^{2+} cation
1	The Arctic, Svalbard archipelago	168.1	19.9	123.9	0.9
2	The Antarctic, Deception island	1 148.1	37.0	103.7	37.0
3	The Antarctic, Galindez island	348.9	17.0	51.0	6.4
4	Ecuador, Papallacta region	169.5	21.2	25.4	0.4
5	Ukraine, Kyiv region	1 265.0	72.3	301.2	21.0
6	Ukraine, “Optymistychna” karst cave	147.0	9.2	64.3	3.7
7	Israel, the Dead Sea	157.9	46.0	105.2	3.3
8	Ukraine, “Atlantida” karst cave (paleosoil)	800	58	0.33	0.66
9	Ukraine, “Atlantida” karst cave (clay)	666.6	6.66	6.66	1

the maximum allowable concentrations in soils (up to 100 ppm Cu^{2+}) [18, 19].

The coefficient of concentration resistance was calculated for the studied ecosystems, which is a quantitative indicator of the adaptation potential of the microbiome (Table 3).

The highest coefficients were shown for microorganisms of Deception Island (#2, Table 3). In the NA medium with Cu^{2+} citrate, it was very high and amounted to as much as 1148. The lowest coefficients were calculated for “Atlantida” cave microbiomes, indicating its low adaptability to Cu^{2+}.

It should be noted that the maximum permissible concentration of Cu^{2+} for the isolated microorganisms was tens and hundreds times higher than the concentration of copper in the samples. The presented results on the coefficients indicate a high adaptive potential of the investigated ecosystems.

The most of isolated CRM interacted with Cu^{2+} compounds. They accumulated and reduced Cu^{2+} to insoluble $Cu_2O\downarrow$. It follows that environmentally promising CRM are able to remove toxic Cu(II) compounds and can be isolated from the natural ecosystems of all geographical zones of the globe. Pure cultures of microorganisms that are resistant to Cu^{2+} in ultra-high concentrations and interact with it were isolated. The isolated cultures were able to remove Cu^{2+} from solutions due to the accumulation in microbial biomass, as well as reduction to insoluble compounds $Cu_2O\downarrow$ (Fig. 6).

The capability of microorganisms to accumulate Cu^{2+} was confirmed by the hydrogen sulfide test [8]. Its essence is the coloration of Cu^{2+} compounds in a dark brown color due to the formation of $CuS\downarrow$. The brown color of the colonies of microorganisms indicated the ability of microorganisms to reduce Cu^{2+} to non-soluble and low toxic $Cu_2O\downarrow$ (Fig. 6). A striking example of the accumulation of Cu^{2+} is shown by the microorganisms of the Deception Island. Mycelial fungi predominated in this sample and intensively accumulated Cu^{2+} (Fig. 7).

Currently, microbial technologies that provide effective wastewater purification and soil bioremediation at Cu^{2+} concentrations above 500 ppm have not been developed [20]. High resistance to Cu^{2+} is shown by the example of strain *Pseudomonas* spp., which is resistant to 300 ppm Cu^{2+} [21] and three strains isolated from the “metal-containing” river Mogpog, resistant to 15–390 ppm Cu^{2+} [22]. It is obvious that for purification of highly copper-contaminated ecosystems (1 000 ppm Cu^{2+} and above) there is a need to isolate more resistant strains. The only mention about high level of bacterial Cu^{2+}-resistance refers to *Thiobacillus ferrooxidans* ATCC, that was 25 000 ppm Cu^{2+} [23]. However, there are no data on the ability of

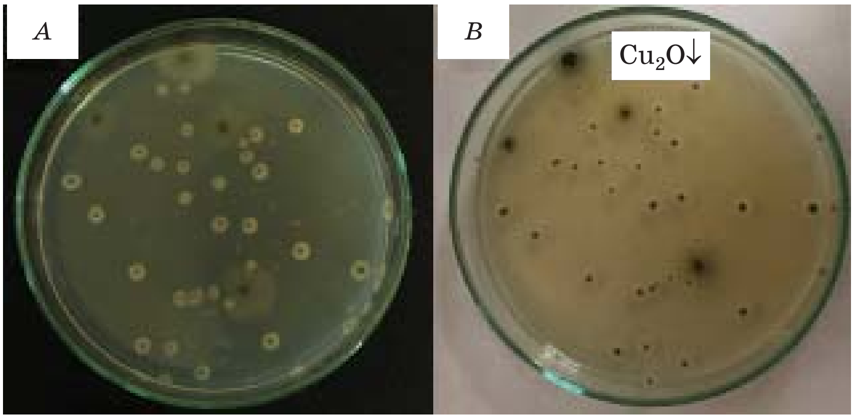

Fig. 7. **Accumulation of Cu^{2+} by mycelial fungi in the MM medium at the presence of 100 ppm Cu^{2+} cation:** *A* — before H_2S addition; *B* — after H_2S addition

this strain to interact with copper and the possibility of its use in bioremediation of ecosystems.

We have previously isolated four bacterial strains of *Pseudomonas* genus with record resistance up to 1.0 M Cu^{2+} (63 546 ppm) from natural ecosystems [24]. They are promising for the purification of super-concentrated metal effluents.

Thus, the soils, clays, sediments and sands of natural ecosystems are a "genetic resource" of copper resistant microorganisms that are promising for development of novel biotechnology of purification of copper-containing wastewater and soil bioremediation.

The isolated microbial cultures are promising for the further application in environmental biotechnologies, for example for copper-containing wastewater purification and contaminated soils bioremediation.

We have confirmed the hypothesis that microorganisms are resistant to toxic Cu(II) compounds in high concentrations up to 15 500 ppm Cu^{2+} and exist in any natural ecosystems containing trace concentrations of toxic Cu(II). Soils, clays, sediments and sands of natural ecosystems of all geographical zones of the globe are a "genetic source" of copper resistant microorganisms. The proposed methodological approach not only allows to isolate copper-resistant microorganisms from natural ecosystems, but also avoid complex genetic transformations in order to obtain perspective genetically modified strains for further application in biotechnologies for purification of industrial wastewater and soil bioremediation.

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REFERENCES

1. *Flemming C. A., Trevors J. T.* Copper toxicity and chemistry in the environment: a review. *Water, Air, & Soil Pollution*. 1989, 44 (1–2), 143–158. https://doi.org/10.1007/BF00228784
2. *Schouten C.* The role of sulphur bacteria in the formation of the so-called sedimentary copper ores and pyritic ore bodies. *Economic Geology*. 1946, 41 (5), 517–538.
3. *Ashida J., Higashi N., Kikuchi T.* An electronmicroscopic study on copper precipitation by copper-resistant yeast cells. *Protoplasma*. 1963, 57 (1–4), 27–32. https://doi.org/10.1007/BF01252044
4. *Dovgalyuk A.* Environmental pollution by toxic metals and its indication by plant test systems. *Studia Biologica*. 2013, 7 (1), 197–204. https://doi.org/10.30970/sbi.0701.269
5. *Masindi V., Muedi K. L.* Environmental contamination by heavy metals. *Heavy Metals*. 2018, P. 115–133. https://doi.org/10.5772/intechopen.76082
6. *Husak V.* Copper and copper-containing pesticides: metabolism, toxicity and oxidative Stress. *J. Vasyl Stefanyk Precarpathian National University*. 2015, 2 (1), 38–50. https://doi.org/10.15330/jpnu.2.1.38-50

7. *Andreazza R., Pieniz S., Okeke B. C., Camargo F. A. O.* Evaluation of copper resistant bacteria from vineyard soils and mining waste for copper biosorption. *Brazil. J. Microbiol.* 2011, 42 (1), 66–74. https://doi.org/10.1590/S1517-83822011000100009
8. *Hovorukha V., Havryliuk O., Tashyreva H., Tashyrev O., Sioma I.* Thermodynamic substantiation of integral mechanisms of microbial interaction with metals. *Ecological Engineering and Environment Protection*. 2018, V. 2, P. 55–63, https://doi.org/10.32006/eeep.2018.2.5563.
9. *Kibria G.* Trace/heavy metals and its impact on the environment, biodiversity and human health– a short review. *Proj. Rech.* 2016, P. 1–5. https://doi.org/10.13140/RG.2.1.3102.2568
10. *Stenberg B., Johansson M., Pell M., Sjödahl-Svensson K., Stenström J., Torstensson L.* Microbial biomass and activities in soil as affected by frozen and cold storage. *Soil Biol. Biochem.* 1998, 30 (3), 393–402. https://doi.org/10.1016/S0038-0717(97)00125-9
11. *Baker T. H. W.* Transportation, preparation, and storage of frozen soil samples for laboratory testing. *Soil Specimen Preparation for Laboratory Testing*. D. A. Sangrey, R. J. Mitchell, Eds. *West Conshohocken, PA: ASTM International.* 1976, P. 88–112.
12. Public Health England. Detection and enumeration of bacteria in swabs and other environmental samples. *National Infection Service Food Water and Environmental Microbiology Standard Method.* 2017, 4 (4).
13. *Ogunfowokan A. O., Adekunle A. S., Oyebode B. A., Oyekunle J. A. O., Komolafe A. O., Omoniyi-Esan G. O.* Determination of heavy metals in urine of patients and tissue of corpses by atomic absorption apectroscopy. *Chemistry Africa*. 2019, 2 (4), 699–712. https://doi.org/10.1007/s42250-019-00073-y
14. *Prekrasna I. P., Tashyrev O. B.* Copper resistant strain *Candida tropicalis* RomCu5 interaction with soluble and insoluble copper compounds. *Biotechnol. acta*. 2015, 8 (5), 93–102. https://doi.org/10.15407/biotech8.05.093
15. *Samanovic M. I., Ding C., Thiele D. J., Darwin K. H.* Copper in microbial pathogenesis: meddling with the metal. *Cell Host Microbe*. 2012, 11 (2), 106–115, https://doi.org/10.1016/j.chom.2012.01.009
16. *Borkow G., Gabbay J.* Copper as a biocidal tool. *Current Medicinal Chemistry*. 2005, 12 (18), 2163–2175. https://doi.org/10.2174/0929867054637617
17. *Havryliuk O., Hovorukha V., Tashyrev O.* The resistance of chernozem soil microorganisms to soluble copper compounds. *Factors in Experimental Evolution of Organisms*. 2018, V. 3826, P. 273–278. (In Ukrainian).
18. *Kisel V. I.* Soil pollution by heavy metals. Agroecological assessment of Ukrainian lands and placement of agricultural crops. *Kyiv: Agricultural Science.* 1997, 160 p. (In Russian).
19. *Mineev V. G.* Chemicalization of agriculture and natural environment. *Moscow: Agropromizdat*. 1990. (In Russian).
20. *Ochoa-Herrera V., León G., Banihani Q., Field J. A., Sierra-Alvarez R.* Toxicity of copper(II) ions to microorganisms in biological wastewater treatment systems. *Science of the Total Environment*. 2011, V. 412–413, P. 380–385. https://doi.org/10.1016/j.scitotenv.2011.09.072
21. *Rajbanshi A.* Study on heavy metal resistant bacteria in Guheswori Sewage Treatment Plant. *Our Nature*. 2009, V. 6. https://doi.org/10.3126/on.v6i1.1655
22. *Parungao M.* Biosorption of copper, cadmium and lead by copper-resistant bacteria isolated from Mogpog River, Marinduque. *Philippine J. Sci*. 2007, 136 (2), 155–165.
23. *Brahmaprakash G. P., Devasia P., Jagadish K. S., Natarajan K. A., Ramananda Rao G.* Development of *Thiobacillus ferrooxidans* ATCC 19859 strains tolerant to copper and zinc. *Bulletin of Materials Science*. 1988, 10 (5), 461–465. https://doi.org/10.1007/BF02744659
24. *Havryliuk O., Hovorukha V., Patrauchan M., Youssef N. H., Tashyrev O.* Draft whole genome sequence for four highly copper resistant soil isolates *Pseudomonas lactis* strain UKR1, *Pseudomonas panacis* strain UKR2, and *Pseudomonas veronii* strains UKR3 and UKR4. *Current Research in Microbial Sciences*. 2020, V. 1, P. 44–52. https://doi.org/10.1016/j.crmicr.2020.06.002

КІЛЬКІСНІ ПОКАЗНИКИ РОЗПОДІЛЕННЯ МІДЬРЕЗИСТЕНТНИХ МІКРООРГАНІЗМІВ У ПРИРОДНИХ ЕКОСИСТЕМАХ

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Мідь є дуже токсичним металом, що розповсюджений як у природних, так і в техногенних екосистемах. Метою роботи було визначити рівень стійкості мікроорганізмів природних екосистем до катіонної форми та органометалічного комплексу Cu^{2+}. Було досліджено мікроорганізми 9 природних екосистем п'яти географічних зон Антарктики, Арктики, Мертвого моря (Ізраїль), середніх широт України та екваторіальної зони Південної Америки (Еквадор). Стійкість мікроорганізмів визначали культивуванням у середовищі з концентраційним градієнтом Cu^{2+}. Кількість Cu^{2+}-резистентних мікроорганізмів у природних екосистемах визначали підрахунком колоній на поживному агарі у присутності цитрату та катіонної форми Cu^{2+}. Концентрацію Cu(II) у зразках ґрунту та глини визначали методом атомно-абсорбційної спектрометрії. Підтверджено гіпотезу про те, що стійкі до Cu^{2+} мікроорганізми існують у будь-якій природній екосистемі. Стійкість до Cu^{2+} у формі катіону була у 8–31 та 14–140 разів нижчою ніж до цитрату Cu^{2+} у живильному та мінеральному агаризованих середовищах відповідно. Кількість Cu^{2+}-резистентних мікроорганізмів у природних екосистемах досягала сотень та тисяч у присутності 175–15 500 мг/л Cu^{2+}. Таким чином, ґрунти, глини та піски природних екосистем є «генетичним ресурсом» мідьрезистентних мікроорганізмів, перспективних для розроблення новітніх біотехнологій очищення мідьвмісних стічних вод та біоремедіації ґрунтів.

Ключові слова: забруднення міддю, мідьрезистентні мікроорганізми, природні екосистеми, природоохоронні біотехнології.

КОЛИЧЕСТВЕННЫЕ ПОКАЗАТЕЛИ РАСПРЕДЕЛЕНИЯ МЕДЬ-РЕЗИСТЕНТНЫХ МИКРООРГАНИЗМОВ В ЕСТЕСТВЕННЫХ ЭКОСИСТЕМАХ

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Медь является очень токсичным металлом, который распространен как в естественных, так и в техногенных экосистемах. Целью работы было определение уровня устойчивости микроорганизмов природных экосистем к катионной форме и органо-металлическому комплексу Cu^{2+}. Были исследованы микроорганизмы 9 природных экосистем пяти географических зон Антарктики, Арктики, Мертвого моря (Израиль), средних широт Украины и экваториальной зоны Южной Америки (Эквадор). Устойчивость микроорганизмов определяли культивированием в среде с концентрационным градиентом Cu^{2+}. Количество Cu^{2+}-резистентных микроорганизмов в естественных экосистемах определяли подсчетом колоний на питательном агаре в присутствии цитрата и катионной формы Cu^{2+}. Концентрацию Cu(II) в образцах почвы и глины определяли методом атомно-абсорбционной спектрометрии. Подтверждена гипотеза о том, что устойчивые к Cu^{2+} микроорганизмы существуют в любой естественной экосистеме. Устойчивость к Cu^{2+} в форме катиона была в 8–31 и 14–140 раз ниже, чем у цитрата Cu^{2+} в питательной и минеральной агаризованных средах соответственно. Количество Cu^{2+} резистентных микроорганизмов в естественных экосистемах достигала сотен и тысяч в присутствии 175–15 500 мг/л Cu^{2+}. Таким образом, почвы, глины и пески естественных экосистем являются «генетическим ресурсом» медь-резистентных микроорганизмов, перспективных для разработки новейших биотехнологий очистки медьсодержащих сточных вод и биоремедиации почв.

Ключевые слова: загрязнение медью, медь-резистентные микроорганизмы, естественные экосистемы, природоохранные биотехнологии.