UDC 579.26

O.B. Tashyrev[1], Ie.P. Prekrasna[1], G.O. Tashyreva[1], O.Iu. Bielikova[2]

[1] Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, academic Zabolotny st, 154, Kyiv, MSP, D03680, Ukraine
[2] National Aviation University, cosmonaut Komarov ave., 1, Kyiv, 03680, Ukraine

RESISTANCE OF MICROBIAL COMMUNITIES FROM ECUADOR ECOSYSTEMS TO REPRESENTATIVE TOXIC METALS - CrO_4^{2-}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}

Microbial communities of the Ecuadorian Andes and volcano Tungurahua were shown to be super resistant to representative toxic metals. Maximum permissible concentrations of toxic metals were 100 ppm of Hg^{2+}, 500 ppm of Co^{2+} and Ni^{2+}, 1000 and 1500 ppm of Cr(VI), 10000 and 20000 ppm of Cu^{2+}. The effect of metal concentration increasing on the biomass growth, CO_2 and H_2 synthesis was investigated. Two types of response of microbial communities on the increasing of toxic metals concentrations were discovered. The first type of response is the catastrophic inhibition of microbial growth. The second type of response is the absence of microbial growth inhibition at certain metal concentration gradient. The succession of qualitative structure of Ecuadorian microbial communities was shown for the first time. Bacteria, yeasts and finally fungi consistently dominate in the microbial community at the Cu^{2+} concentration raising. Microorganisms resistant to ultra-high concentrations of toxic metals (e.g., 3000 ... 20000 ppm of Cu^{2+}) were isolated from Ecuadorian ecosystems. These microorganisms are able to accumulate toxic metals.

Key words: microbial communities of Ecuador, microbial resistance to metals.

Microbial communities play a key role in the global cycles of elements and distribution of vector fluxes of carbon and energy in the biosphere. Obviously, the stability of the microbial communities (hereinafter - MC) depends on their homeostasis. As homeostasis we mean ability of MC to keep stable functioning in the presence of extreme factors and to overcome their negative effects.

The variety of extreme factors can be divided to the following "classes":

1. electromagnetic radiation (ultraviolet radiation, α-, β-, γ-radiation);
2. metal and nonmetal inorganic compounds;
3. synthetic organic xenobiotics (nitro-, chlore-, aromatic and other derivatives);
4. natural organic compounds, which in high concentration are toxic (e.g., solid food waste).

Metals have the strongest negative effect on MC among the second class of extreme factors. Several so-called representative metals can be highlighted out of 48 metals included in the periodic system of elements. These representative metals integrate all known mechanisms of the negative effects of metals on microbes.

We have picked out the following groups of metals:

1. Metals-oxidants. The negative effects of such metals are based on their high redox potential. Such metals oxidize structural components of microbial cells, block the number of enzymes of both constructive and energy metabolism. The above-mentioned metals are CrO_4^{2-}, VO^{3-}, MoO_4^{2}, Tc^{3+}, etc.

2. Metals-substituents. The negative effect of metal-substituents is caused by the replacement of macro elements by toxic metals in the structural components of microbial cells and in the active sites of enzymes. Such metals as Co^{2+}, Ni^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, etc have this negative effect on microbial cells.

3. Metals of combined action. This group includes metals that combine properties of both metals-oxidants and metals-substituents: Hg^{2+}, Cu^{2+}, etc.

This classification of toxic metals enables to avoid complex and time-consuming researches of MC` resistance to dozens of toxic metals. Therefore, we have chosen as representative the

following metals: CrO_4^{2-} (metal-oxidant), Co^{2+}, Ni^{2+} (metals-substituents), Hg^{2+} and Cu^{2+} (metals of combined action) to investigate resistance of microbial communities from Ecuador to toxic metals. Quantity parameter of microbial resistance to toxic metals was maximal permissible concentration (MPC) of metals. As toxic metals primarily inhibit the microbial growth and metabolism, we investigated the metal influence on the biomass growth and gas phase composition as well.

Resistance of the MC to the metals largely depends on the ability of microorganisms to interact with toxic metals, which usually leads to detoxification of the metals. Microorganisms are able to deposit metals with microbial metabolites, accumulate them in biomass, reduce them to insoluble forms etc. Therefore, in this study we determined not only the maximum permissible concentrations of metals (MPC) for Ecuadorian microorganisms, but also investigated the interaction of these microorganisms with metals.

The aim of the study is to investigate the influence of representative toxic metals on the MC of Ecuador ecosystems and to define the quantitative parameters of microbial resistance to metals.

The objects of study are the microbial communities of Ecuador ecosystems: soil from the rocky pockets of Andes cliff and ashes of the active volcano Tungurahua.

Materials and methods. *Sampling.* Soil samples were collected in the Ecuadorian Andes (mountain range Cordillera Real, height 4030 m) on October 31. The samples were collected from the rocky pockets of cliffs with the height 30 m near Papallacta, Napo Province. The samples of ashes were collected on 4 November 2013 on the active volcano Tungurahua. The sampling points are shown on Fig. 1. The process of sampling and a general view of the cliff and the volcano are shown on Fig. 2. Samples were kept in sealed plastic bags after collecting.

Fig. 1. Points of sampling. 1 – cliff near Papallacta; 2 – volcano Tungurahua.

Fig. 2. Sampling in ecosystems of Ecuador, A – cliff near Papallacta; B – sampling of soil from the cliff rocky pockets; C – volcano Tungurahua; D – sampling at the volcano Tungurahua.

Preparing of metals solutions. Stock solutions of Cr(VI), Ni^{2+} and Hg^{2+} contained 10,000 ppm of metal ions. Stock solutions of Co^{2+} and Cu(II) citrate contained 20,000 ppm of metal ions. Stock solutions of Cr(VI), Ni^{2+} and Co^{2+} were obtained by dissolving of metal salts in distilled water (K_2CrO_4, $NiCl_2$ and $CoCl_2$ respectively). Solution of Cu(II) citrate was obtained by dissolving of $CuCl_2$ in aqueous solution of $Na_3C_6H_5O_7$. Solution of Hg^{2+} was obtained by dissolving metallic mercury in nitric acid.

Preparing of metal-containing nutrient medium. Metal solutions were added to nutrient broth (company HiMedia Laboratories Pvt. Ltd., USA) or to the melted and cooled to 45º nutrient agar (company HiMedia Laboratories Pvt. Ltd., USA).

Maximum permissible concentrations (MPC) of toxic metals for microbial community were determined as follows. The soil (100 mg) was placed in a 20 ml tube, and then nutrient broth with metal (10 ml) was added to the tube. Each variant of experiment (i.e., each tube) contained one toxic metal. The microorganisms were cultivated at presence of 100, 500, 1000, 1500 ppm of Cr(VI); 100, 500, 1000 ppm of Ni^{2+} and Co^{2+}; 20, 50, 100, 150 ppm of Hg^{2+}; 500, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000 ... 20 000 ppm of Cu^{2+}. The tubes were closed with conical rubber plugs. Microorganisms were cultivated at 28°C. The maximum concentration of metals, where the growth of microorganisms was observed, was accepted as MPC.

Strains of metal resistant microorganisms were isolated from batch metal containing cultures. Culture liquid was inoculated by the loop on the surface metal containing medium and cultivated at 28°C. The isolates were passaged on the solid metal containing medium until the homogeneous colonies of microorganisms with identic cells were obtained.

Biomass growth of microorganisms in liquid culture was determined by the change of optical density at the photoelectric colorimeter KFK 2-MP at $\lambda = 540$ nm, the length of optical step = 0,5 cm.

Composition of the gas phase. Composition of the gas (H_2, O_2, N_2, CO_2) in the cultivator was determined on the seventh day of the cultivation of microorganisms. Plastic sterile 2,5 ml syringes (company «Bayer») with a rubber seal on the piston were used for gas sampling.

The gas composition was determined by standard method based on the thermal conductivity of the katharometer on gas chromatograph LHM-8-MD. Two steel columns were used. The first one (I) was for the analysis of H_2, O_2, N_2 and CH_4, the second one (II) was for the analysis of CO_2.

Parameters of columns: I - l = 3, m, d = 3 mm, the sorbent 13X (NaX); II - l = 2, m, d = 3 mm, the sorbent Porapak-Q. The temperature of columns is +60 °C, the temperature of evaporator is +75 °C and of detector +60 °C. The detector current is 50 mA. Gas carrier is argon; gas flow rate is 30 ml/min. The concentration of gases (H_2, O_2, N_2 and CO_2 in %) was calculated according to the peak areas.

Contrasting of Hg^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} in microbial biomass with H_2S. Hydrogen sulfide reacts with divalent metal cations with black or dark-brown insoluble metals sulphides formation: $Me^{2+} + S^{2-} = MeS\downarrow$. This reaction was used to detect, whether microorganisms had accumulated divalent metals. Under a fume hood 6 ml of H_2S were added via syringe to the tube, where microorganisms had being grown on the agar sloped surface in the presence of metals. The biomass that had accumulated divalent metals turned intense dark-brown color.

Measuring of the redox potential. Redox potential was determined with the pH-meter-Milivoltmeter "pH-121" (or "EV-74") with the electrodes: a platinum measuring electrode EPV-1, glass electrode ESL- 63-07 (for pH measurement) and flow-silver chloride reference electrode EVL-1MZ.

Results and discussion. Microbial communities of Andes` cliffs and volcano Tungurahua ashes are super-resistant to representative toxic metals (CrO_4^{2-}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}). Thus, maximal permissible concentrations (MPC) of toxic metals were as follows: 50 ppm of Hg^{2+}, 500 ppm of Co^{2+} and Ni^{2+}, 1000 ppm of Cr(VI) and 20 000 ppm of Cu^{2+} for microbial community of Andes` cliffs (Fig. 3A). The MPC for microbial community of volcano Tungurahua ashes were 100 ppm of Hg^{2+}, 500 ppm of Co^{2+} and Ni^{2+}, 1500 ppm of Cr(VI) and 10000 ppm of Cu^{2+} (Fig. 3B). At these concentrations the biomass growth was observed (Fig. 4). Microscopy of "squashed drop" of the culture liquid revealed alive actively moving cells of microorganisms.

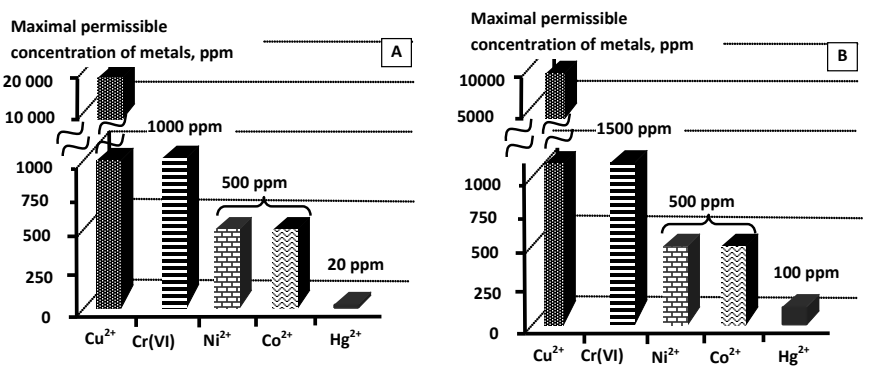

Figure 3. Maximum permissible concentrations of representative toxic metals for microbial communities of Ecuador ecosystems; A – microbial community of Andes; B – microbial community of volcano ash.

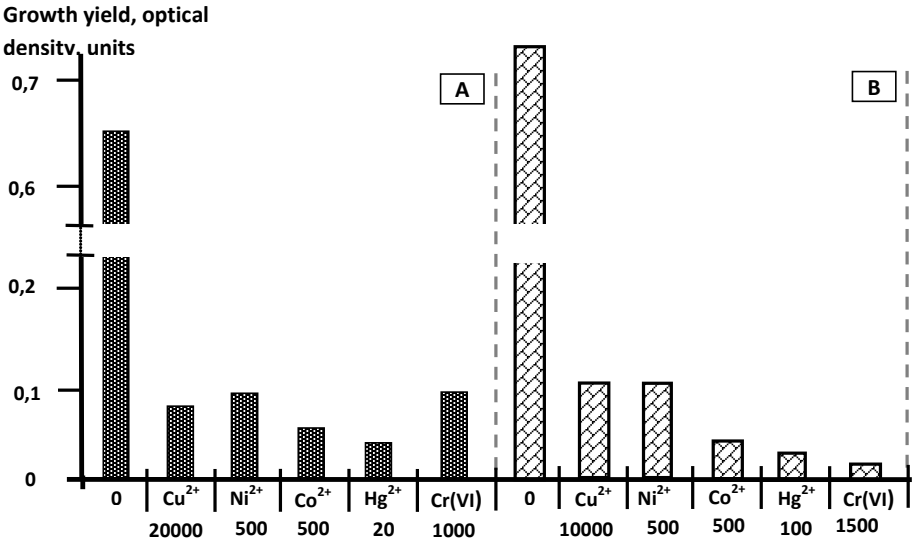

Fig. 4. Influence of MPC of toxic metals on the growth of microorganisms from extreme Ecuador ecosystems; A – microbial community of Andes; B – microbial community of volcano ash.

According to the obtained results both microbial communities form the following resistance order:

$$\mathbf{Hg^{2+} < Co^{2+} < Ni^{2+} < Cr(VI) < Cu^{2+}}$$

Microorganisms from volcano ashes were in 5 times more resistant to Hg^{2+} compared to microorganisms from cliffs (100 and 20 ppm of Hg^{2+} respectively). The resistance of volcano microorganisms to Cr(VI) was 1,5 times higher than the resistance of cliff microorganisms (1500 and 1000 ppm of Cr(VI)). But microorganisms from cliffs were twice more resistant to copper (20 000 and 10 000 ppm of Cu^{2+}).

By the way, 1–10 ppm of toxic metals is bactericide for most known water and soil microorganisms (Table 1). Hence, the MPC of metals for Ecuadorian microbial communities overweighed the common bactericide concentration in several orders. For example, MPC of Hg^{2+} for the microbial community of cliffs was 20 ppm, which in 20...40 times exceeds the bactericidal concentration (0,5 – 1,0 ppm of Hg^{2+}). And MPC of Hg^{2+} for microbial community of volcano ashes exceeds bactericide concentration by 100…200 times (MPC is 100 ppm of Hg^{2+}).

Table 1

Bactericidal concentrations of metals and their negative effect on microorganisms

N	Microorganisms	Metal	C-tion, ppm	Negative effect
1	*Micrococcus* sp.	Cu^{2+}	0,02	Growth inhibition [10]
3	*Pseudomonas putida*		0,5	Bactericidal effect [9]
4	Microbial community of water purification system		4,6	Inhibition of 50% of heterotrophic microorganisms [12]
5	*Erwinia herbicola*	Hg^{2+}	1	Death of cells after 4 hours of exposition [7]
6	*Agrobacterium tumefaciens*		1	Death of cells [7]
7	*Torulopsis glabrata*	Ni^{2+}	1	Growth inhibition [6]

Though, the studied microorganisms are super resistant to toxic metals, the following biological tendency was observed. Microbial growth was inevitably inhibited when metals concentration increased. Mercury was the most toxic for the microbial community of the cliffs. Thus, the inhibiting coefficient ***K*** was 19,1 at the presence of 20 ppm of Hg^{2+}. Volcano microorganisms were more resistant to Hg^{2+}. The inhibition coefficient ***K*** was 20,8 at 100 ppm of Hg^{2+} (MPC of Hg^{2+}), i.e. growth of biomass decreased in 20,8 times compared to control (Fig. 4, A, B).

Cobalt and nickel are stereochemical analogues (ionic radiuses are 0,07 nm). So, these metals have the same mechanism of microbial growth inhibition. However, Co^{2+} was in 6 times more toxic to the cliff microbial community than Ni^{2+}. The inhibition coefficient ***K*** was 39,2 at 500 ppm of Co^{2+} and 6,4 at 500 ppm of Ni^{2+}. Cobalt was twice more toxic than nickel for volcano ashes microbial community. Thus, the inhibition coefficient ***K*** was 16,6 at 500 ppm of Co^{2+} and 7,4 at 500 ppm of Ni^{2+} (Fig. 4, A,B).

Chromium(VI) in dozens of times inhibited growth of microorganisms from both ecosystems. The inhibition coefficient ***K*** for cliff microbial community was 67,0 at 1000 ppm of Cr(VI). And its value for volcano ash microorganisms was 73,0 at 1500 ppm of Cr(VI) (Fig. 4, A, B).

Studied MC were the most resistant to Cu^{2+}. Even at 20 000 ppm of Cu^{2+} the growth of cliff microorganisms was inhibited only in 9,8 times. Growth of volcano microorganisms was inhibited in 8,1 times at 10 000 ppm of Cu^{2+} (Fig. 4, A, B). Bactericidal concentration of Cu^{2+} for the majority of chemoorganotrophic microorganisms is in the range of 1-10 ppm (Table 1). For example, the growth of *Micrococcus* sp. was inhibited already at 0,02 ppm of Cu^{2+}, and *Pseudomonas putida* was inhibited at 0,5 ppm of Cu^{2+}. Maximal permissible concentrations of Cu^{2+} for Ecuadorian microorganisms outreached these concentrations by 20000–1 000000 times. Moreover, Ecuadorian microorganisms were more resistant to copper comparatively with previously isolated metal resistant Antarctic microorganisms [4]. For example, *Brevibacterium antarcticum* B-3204 isolated from soil of Is. Galindez was resistant to 1000 ppm of Cu^{2+}. However, its biomass growth was inhibited in 32 times at this concentration (Fig. 5).

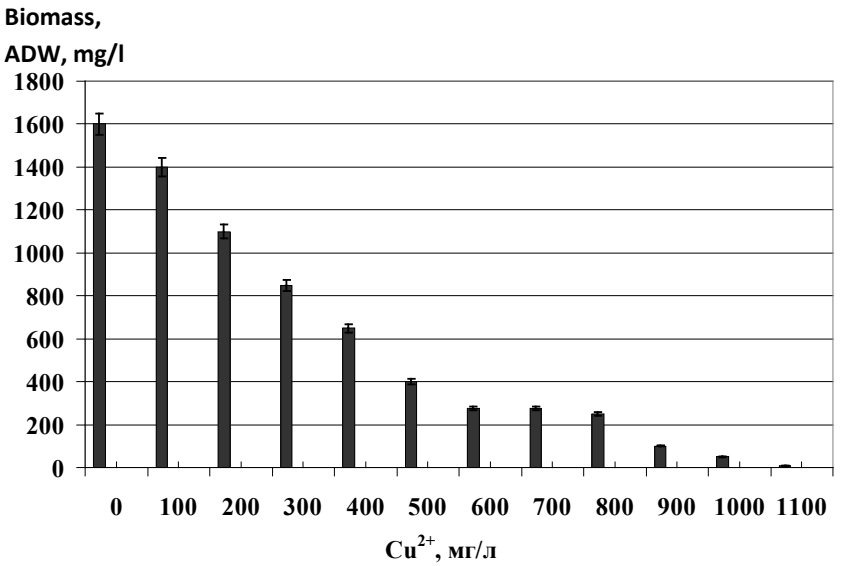

Fig. 5. Dependence of *Brevibacterium antarcticum* growth on the Cu^{2+} concentration [5].

Logically the question arises, what caused such high resistance of Ecuadorian microorganisms to copper. According to our calculations, growth of microorganisms at very high concentrations of toxic metals is thermodynamically allowed [1, 3]. All reactions of energy metabolism are possible only in the zone of thermodynamic stability of water. This zone is limited by two redox reactions (Fig. 6):

$2H_2O = O_2 + 4H^+$ $E_o = +814$ mV (Fig. 6, reaction a)

$2H^+ + 2e = H_2$ $E_o = -414$ mV (Fig. 6, reaction b)

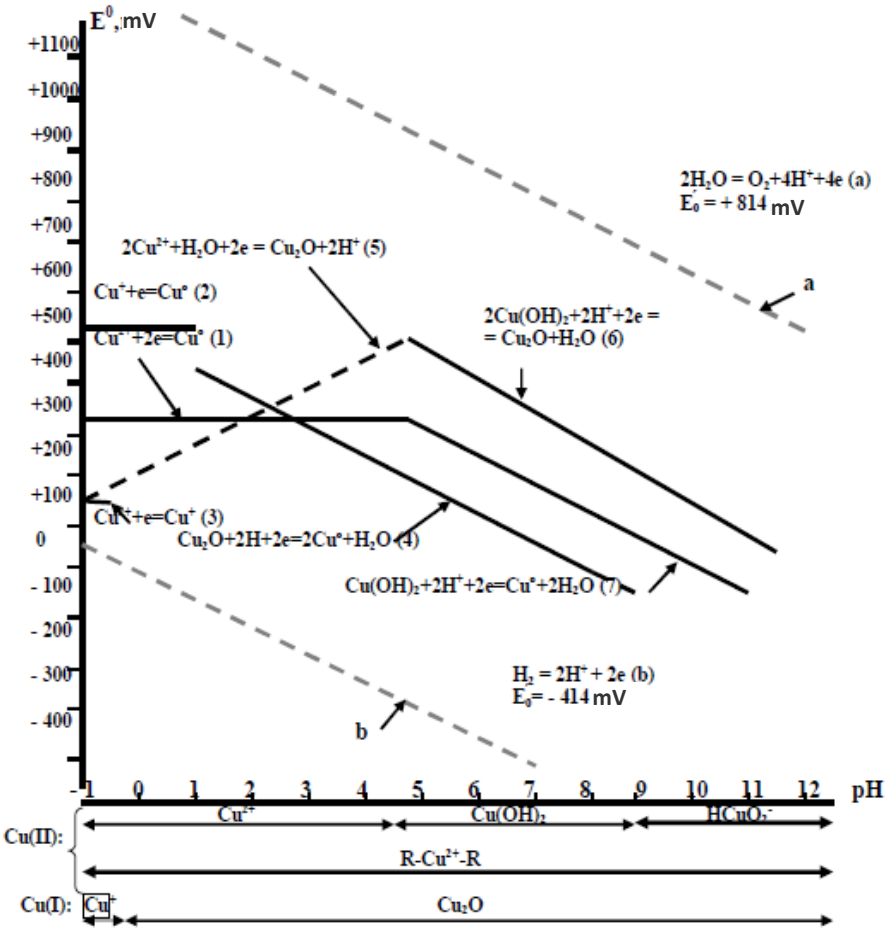

Fig. 6. The redox state of copper at 1 M Cu(II).

Outside the limits of these reactions the water is oxidized with O_2 formation (reaction ***a***) or reduced to H_2 (reaction ***b***) [13]. Obviously, the microorganisms provide reactions of energy metabolism only in the zone of thermodynamic stability of water, i.e. inside the limits of the reactions ***a*** and ***b***. Redox potentials of all of reactions of copper(II) reduction at 1 M of Cu(II) are within the zone of thermodynamic stability of water (Fig. 6). So, microorganisms are able to exist even at such high concentration copper (1,0 M of Cu^{2+} = 63,54 g/l of Cu^{2+}). For example, the redox potential of reaction 5 (reduction of Cu^{2+} to Cu_2O) at 1 M of Cu^{2+} is +474 mV, which is for 340 mV lower than the potential of water oxidation (reaction ***a***, $E_{o\square}$ = +814 mV). Therefore, from the standpoint of thermodynamic prognosis of microbial interaction with copper there is no prohibition against microbial growth even in one molar concentration of copper(II).

Growth of Ecuadorian microbial communities in the presence of ultrahigh concentrations of copper confirms our thermodynamic calculations. Growth of the cliff microbes in the presence of 10 000 ppm of Cu^{2+} was observed after 5 days of cultivation. At 20 000 ppm of Cu^{2+} gas bubbles began to form on the forth day and microbial biofilm formed on the surface of culture liquid on 7 day. Microbial community of volcano ash was also super resistant to Cu^{2+}. The microbial growth was detected on the first day of cultivation in the control (without metals). In the presence of 500-5000 ppm of Cu^{2+} growth was observed on the 2 day. Lag phase increased by 10 times comparing to control in the presence of 10 000 ppm of Cu^{2+}. Thus, filamentous fungi started to grow after 10 days of cultivation, and bacteria appeared in the culture liquid on the 15 day. Minimal delay of growth in the presence of ultra-high metal concentrations also indicates high resistance of Ecuador microbial communities to representative toxic metals.

It is known that filamentous fungi are more resistant to the toxic metals compared to bacteria. Their rapid growth is usually observed even at 500-1000 ppm of Cu^{2+}. In the presence of toxic metals "bacterial link" of microbial community is replaced and the dominant position is occupied by fungi mainly of *Penicillium*, *Mortierella* and *Aspergillus* genera [16, 17]. A similar pattern was shown on the example of Antarctic microorganisms [4]. Thus, the growth of the most resistant to copper Antarctic strain *B. antarcticum* B-3204 was dramatically inhibited at 1100 ppm of Cu^{2+}. And only single colonies of filamentous fungi were isolated from Antarctic soils at higher concentrations (1200 ppm of Cu^{2+} and more). Taxonomic composition of Ecuador MC changes in a different way, when copper concentration increases.

Bacteria occupied the dominant position in the Andes cliff MC at 500...1000 ppm of Cu^{2+}. At higher concentrations (2000 ... 7000 ppm of Cu^{2+}) the dominant position was occupied by yeast. Filamentous fungi appeared in culture liquid only at 8000 ppm of Cu^{2+} and more.

Bacteria dominated in the range 500...5000 ppm of Cu^{2+} in the microbial community of volcanic ash as well. At 10 000 ppm of Cu^{2+} weak growth of filamentous fungi was detected after 10 days of cultivation. Yeast and bacterial cells were found in the culture liquid on 15 day of cultivation. The ratio of bacteria, yeast and fungi by microscopy of the culture liquid was visually estimated as 5-10% of bacterial cells, 70-80% of yeast cells and 5-10% of fungi.

Thus, when concentration of Cu^{2+} increases, the dominate position in the studied MC is consistently occupied by bacteria, yeasts and fungi. Differently the MC reacted in the presence of other representative metals - Hg^{2+}, Co^{2+}, Ni^{2+} and Cr(VI). "Yeast link" was not detected when concentrations of these metals rose. Filamentous fungi dominated in the microbial communities at concentrations close or equal to the MPC.

Microbial growth is known to be correlatively inhibited when concentration of metal in the medium raises. However, we have found two types of microbial "response" on the increasing of metals concentrations [9]. As response of the first type we mean inhibition of microbial growth correlatively with metal concentration increasing. Response of the first type is well known. The second type of response is the absence of inhibition of microbial growth by metal in the particular concentration range [9]. Response of the first type was shown previously on the example of microbial communities of Antarctic, Negev desert and Dead Sea ecosystems, as well as an example of the strain *B. antarcticum* B-3204. Fig. 5 shows that the biomass growth of *B. antarcticum* B-3204 proportionally decreases with increasing of copper concentration from 0 to 1100 ppm of Cu^{2+}. Response of the first type was also found for the microbial communities of Ecuador ecosystems (Fig. 7 and Fig. 8).

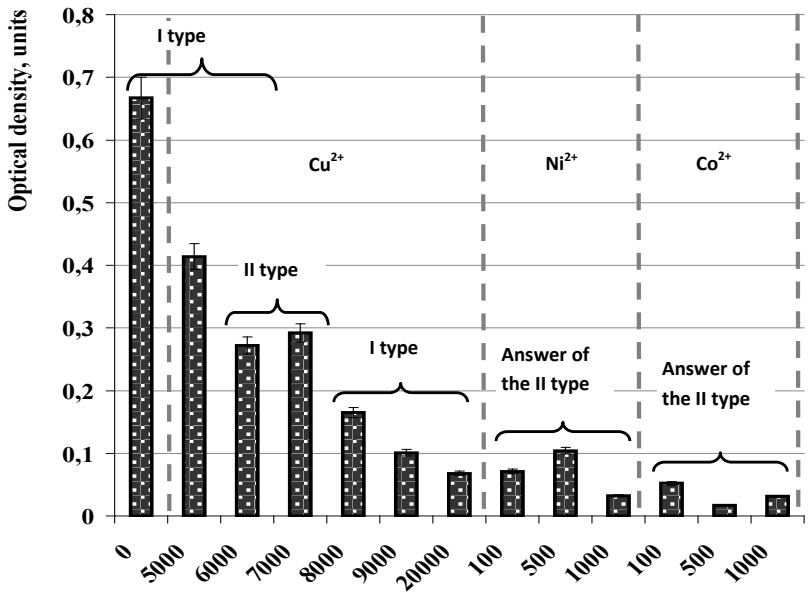

Fig. 7. Two types of response of Andes microbial community to the metals concentration increasing on the example of biomass growth.

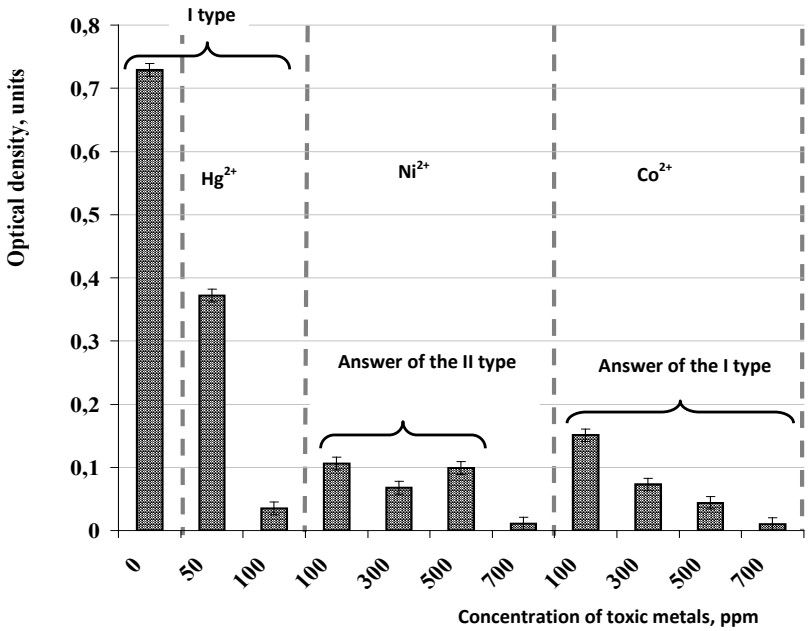

Fig. 8. Two types of response of volcano ashes microbial community to metal concentration increasing on the example of biomass growth.

Fig. 7 shows that while Cu^{2+} concentration increased for 15 000 ppm (from 5000 ppm to 20 000 ppm of Cu^{2+}), biomass yield (optical density) of Andes cliff MC declined in 6 times (from 0,41 to 0,07 units.). Nickel, cobalt and mercury were especially toxic to this MC. Thus, the optical density decreased in 9 times at the presence of 100 ppm of Ni^{2+} (from 0,66 to 0,07 units). Presence of 100 ppm of Co^{2+} in the medium caused inhibition of microbial growth in 12,8

times (from 0,66 to 0,05 units). Mercury dramatically inhibits the growth of microorganisms. Optical density at 20 ppm of Hg^{2+} decreased 19 times comparatively to control (0,66 and 0,03 units respectively).

Similarly, raising the metals concentration caused inhibition of volcano ash MC (Fig. 8). Optical density of the culture liquid falls down 7,3 times comparatively with control (from 0,73 to 0,1 units) at 100 ppm of Ni^{2+}. Value of optical density was 4,8 times lower, than in the control at presence of 100 ppm of Co^{2+} (0, 73 and 0,15 units respectively). Mercury is the most toxic. The presence of 100 ppm of Hg^{2+} decreases the optical density value 18 times (from 0,73 to 0,04 units), i.e. to almost complete inhibition of growth.

There is the second type of response in the MC of Ecuador ecosystems. Fig. 7 shows that optical density values were almost equal (0,29 and 0,27 units) when Cu^{2+} concentration rose from 6000 to 7000 ppm (ΔC [Cu^{2+}] = 1000 ppm). Growth of microorganisms of both MC was not significantly inhibited when concentration of Ni^{2+} rose from 100 to 500 ppm (Fig. 7 and Fig. 8). Thus, the changes of optical density values here are within experimental error. Be noted the sequence of the responses of the first and second type of cliff MC with the Cu^{2+} concentrations increasing (Fig. 7). Whereas the range 0 – 6000 ppm of Cu^{2+} the first type of response was observed, the second type was detected in range 6000 - 7000 ppm of Cu^{2+}. Response of the first type is observed again in the range 7000 - 20000 ppm of Cu^{2+}. The second type of response can be assumed to indicate high adaptive capacity of MC of Ecuador ecosystems.

Gas bubbles formed during the growth of microorganisms in the presence of toxic metals. Analysis of the of the gas phase composition (Fig. 9 and Fig. 10) showed that in all variants of the experiment concentration of CO_2 increased in the range of 1 ... 19%, which indicates a high microbial metabolic activity even at the presence of toxic metals.

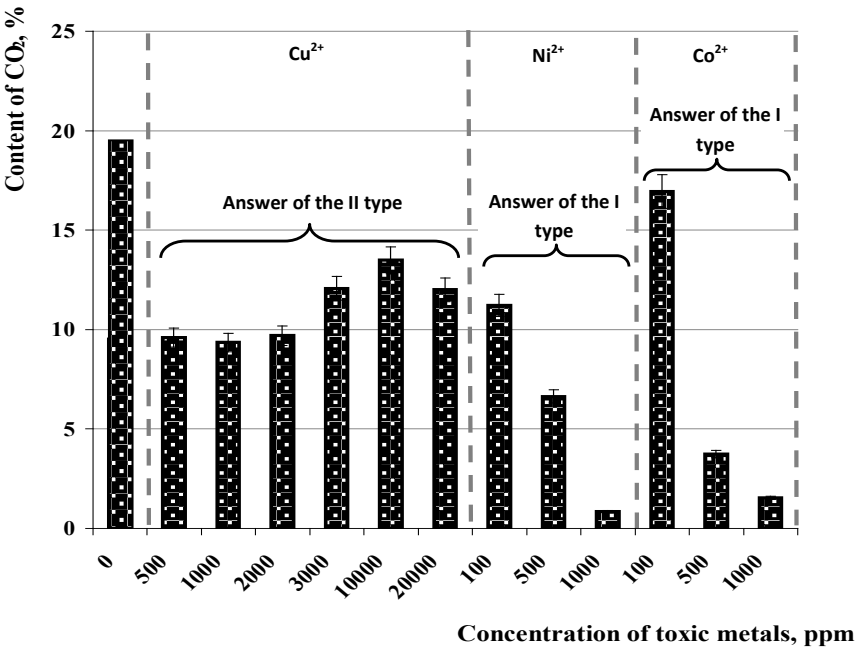

Fig. 9. Two types of response of Andes microbial community to metal concentration increasing on the example of CO_2 synthesis.

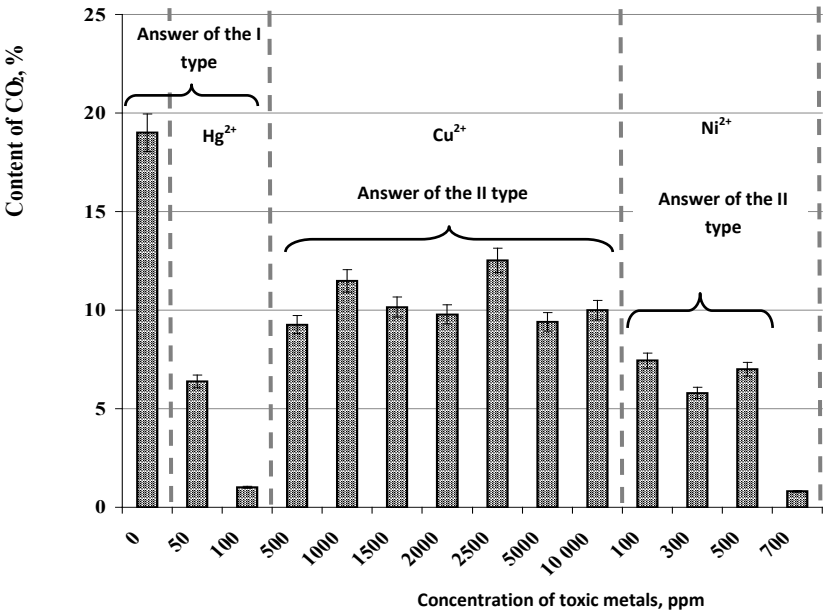

Fig. 10. Two types of response of volcano ash microbial community to metal concentration increasing on the example of CO_2 synthesis.

Carbon dioxide is a common final product of metabolism for all chemoorganotrophic microorganisms. Therefore, the concentration of the CO_2 in the gas phase in this experiment quantifies the level of microbial metabolism inhibition by toxic metals. We have shown two types of response of Ecuador MC on the increasing of metals concentrations on the basis of the CO_2 concentration as well. Thus, increasing of Ni^{2+} and Co^{2+} concentrations from 100 to 1000 ppm (Fig. 9), and Hg^{2+} from 50 to 100 ppm (Fig. 10) leads to a drastic CO_2 content falling down. This is the first type of response. However, CO_2 content did not change considerably when Cu^{2+} concentration rose from 500 to 2000 ppm and Ni^{2+} one from 100 to 500 ppm (Fig. 9). This is a second type of response.

Correlation between such criteria of metabolic activity as biomass growth (Fig. 8) and CO_2 content (Fig. 10) was shown on the example of the volcano ash MC. Thus, both values of optical density and the CO_2 content had almost no changes in range of 100...500 ppm of Ni^{2+}. This means that volcano ash MC gives the second type of response according to the both the criterion of biomass growth and the CO_2 content.

Microbial communities of the both ecosystems synthesized H_2 during growth in a liquid medium in the control and in the presence of metals (Fig. 11 and Fig. 12). For example, MC of a volcanic ash synthesized 0,04% of H_2 at 10,000 ppm of Cu^{2+} and 3% of H_2 at 5000 ppm of Cu^{2+}. Moreover, MC of volcano ash synthesized even trace amounts of H_2 in the presence of Cr(VI), and Hg^{2+}, i.e. metals-oxidizers with high values of redox potential ($E_0 Hg^{2+}$ = +920 mV at pH = 2, E0, $E_0 Cr(VI)$ = +555 mV at pH = 7). Microorganisms of the cliff ecosystem did not synthesize H_2 in the presence of Cr(VI) and Hg^{2+} (Fig. 11).

Microorganisms of cliff ecosystem synthesized less H_2 comparatively to microorganisms of volcanic ash. However, the general pattern for both MC is H_2 forming even at high concentrations of metals with oxidizing properties. Thus, 0,1% of H_2 was found even at 20 000 ppm of Cu^{2+}.

Synthesis of H_2 in the presence of toxic metals is caused, apparently, by the presence of facultative and obligate anaerobic metal resistant microorganisms in MC. Obviously the more diverse microbial community is, the faster it can restore its stabile functioning in the presence of extreme factors. Potential presence of anaerobic metal resistant microorganisms indicates

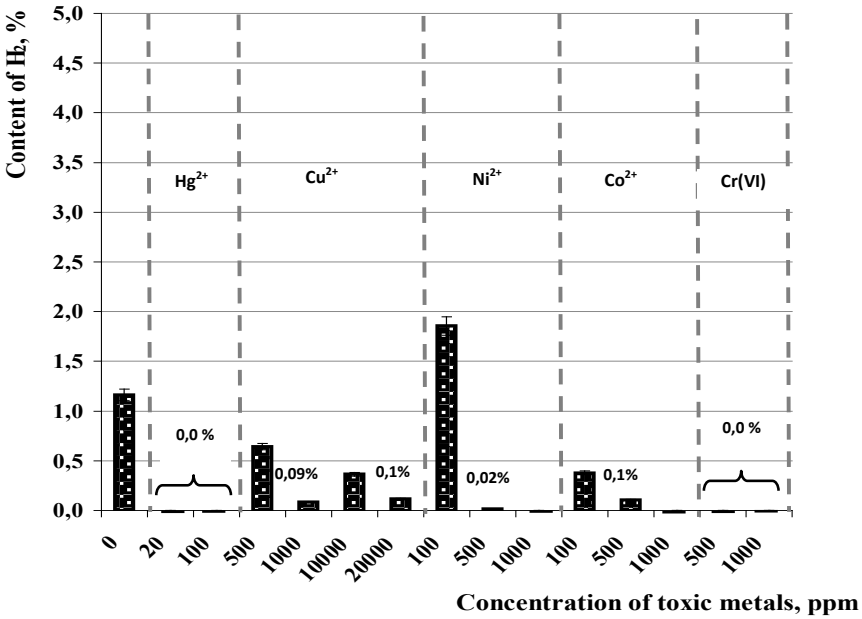

Fig. 11. Synthesis of H_2 at the presence of toxic metals by Andes microbial communities.

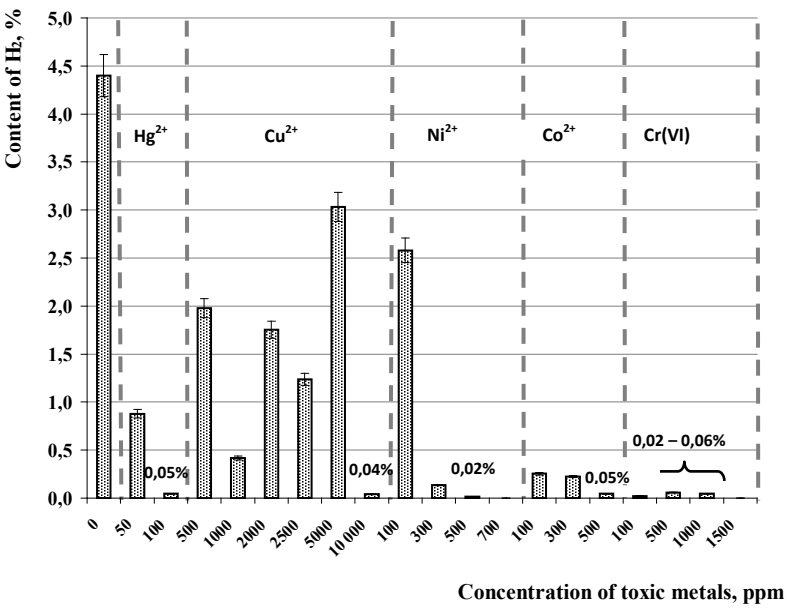

Fig. 12. Synthesis of H_2 at the presence of toxic metals by volcano ash microbial communities.

extremely high stability of studied MC in presence of toxic metals. However, the MC of volcano ash was more resistant to metals-oxidizers, because anaerobic organisms were metabolically active even in the presence of 1000 ppm of Cr(VI) and 100 ppm of Hg^{2+}.

Synthesis of H_2 occurs due to the activity of dehydrogenase enzymes, which reduce redox potential to -400…-420 mV. However, synthesis of hydrogen took place in the presence of high potential toxic metals – Cu^{2+}, Hg^{2+} and Cr(VI). In the range 500...20 000 ppm of Cu^{2+} redox potential is +330...+ 360 mV, at 500...1500 ppm of Cr (VI) redox potential is +320...+ 350 mV.

And at 50...100 ppm of Hg^{2+} the redox potential value is +340...+380 mV. The question arises, how anaerobic microorganisms could survive at such high redox potential. The answer can be as follows. Soil particles (solid phase) and a liquid nutrient medium with toxic metals form heterophase system. One can assume that the soil particles create a protective layer for anaerobic microorganisms. There metal resistant anaerobic microorganisms can create local areas and synthesize H_2. Nevertheless, anaerobic microorganisms have to overcome very tough conditions, as H_2 content is trace.

Content of H_2 falls drastically, when Ni^{2+} and Co^{2+} concentrations increase from 100 to 500 ppm. This tendency is shown for both MC (Fig. 11 and Fig. 12). Thus, synthesis of H_2 by Andes cliff MC falls from 1,86% to 0,02% when Ni^{2+} concentration increased. Synthesis of H_2 by MC of volcanic ash decreased from 2,6% to 0,02%. As concentration of Co^{2+} raised synthesis of H_2 by cliff microorganisms dropped from 0,4% to 0,1%. The same trend is for MC of volcanic ash. The H_2 content falls from 0,26% to 0,05%. Nickel and cobalt are metals-substituents. The core of their damage for microorganisms lies in metals replacing macro elements in structural components of microbial cells. Ionic radii of Co^{2+} and Ni^{2+} (0,07-0,08 nm) are equal to ionic radius of Fe^{2+} (Fig. 13), which is the active center of the enzyme ferredoxin [2]. We can assume that the nickel and cobalt ions replace Fe^{2+} in the molecule of ferredoxin, and thereby block the electron transport chain of anaerobic microorganisms. According to the values of biomass growth (Fig. 7 and Fig. 8), Co^{2+} is more toxic to microorganisms compared to Ni^{2+}. However, both cations are «blockers» of hydrogen-synthesizing enzymes.

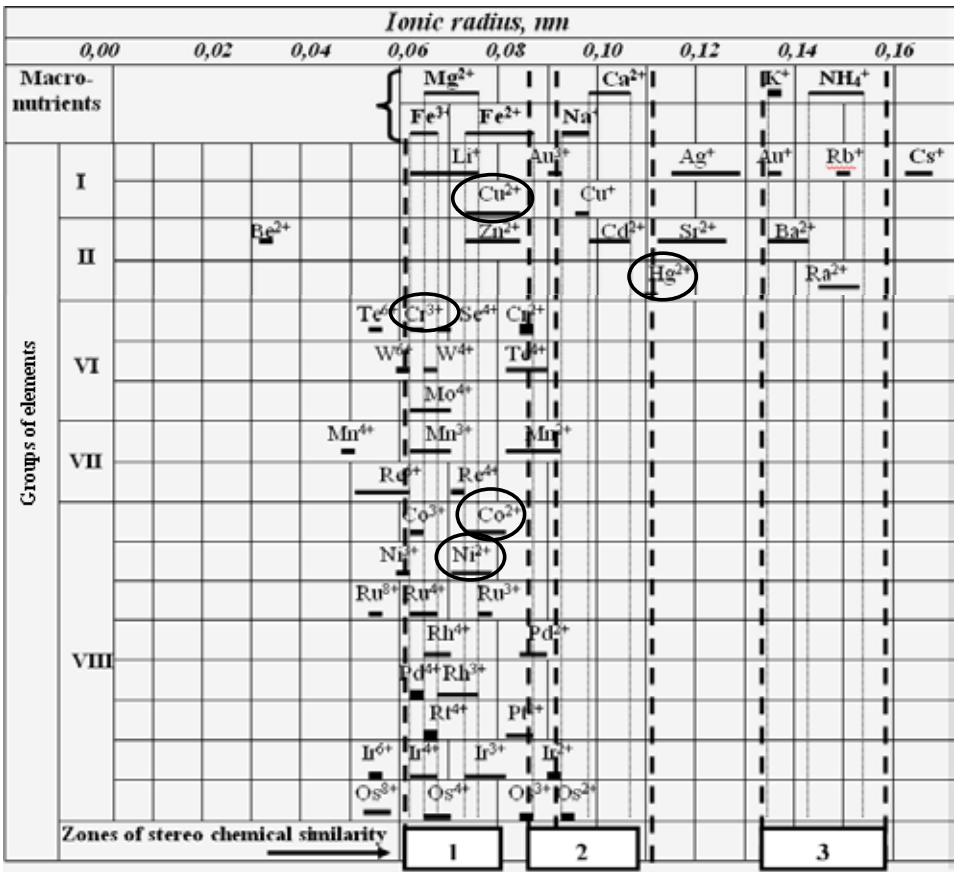

Fig. 13. Stereochemical analogy of toxic metals and macronutrients.

Increasing the concentration of Cu^{2+}, in contrast, does not suppress the H_2 synthesis. Probably, microorganisms of Ecuador ecosystems are able to prevent the penetration of copper cations into the cell. There are several mechanisms of resistance to copper: reduction of Cu^{2+} to insoluble nontoxic compounds, binding metals with microbial metabolites, accumulation in the cell wall etc. For example, as copper is a metal with oxidizing properties, microorganisms can reduce Cu^{2+} to the insoluble and accordingly non-toxic Cu_2O or Cu^0 on the surface of the cytoplasmic membrane [15]. Copper may form insoluble complexes with microbial exometabolites, for example $CuCO_3$, CuS, copper-protein complexes, complexes of copper with exopolysaccharides [11]. Copper is a stereochemical analogue of Mg^{2+} (ionic radius 0,07-0,08 nm, figure 13), so it may replace Mg^{2+} in the murein and accumulate in cell wall.

Known fact is that metal resistant pure cultures of microorganisms are promising for biotechnologies of metal waste water and soils treatment. So, metal resistant strains were isolated out of Ecuador ecosystems. Some of these strains were able to interact with toxic metals, in particular, to accumulate it in the biomass. Thus, 14 strains were isolated out Andes cliff ecosystem, 12 of which accumulated toxic metals (Table 2).

Table 2

Metal resistant microorganisms isolated from Ecuador ecosystems

	Strain	Morphology of the colony	Morphology of the cells	Gram stain	Metal accumulation in biomass
Andes, Ecuador	RomCu1	Grey, circular, shiny, smooth, raised	rod	G-*	Cu(II)
	RomCu2	Beige, circular, shiny, smooth, raised	rod	G-	Cu(II)
	RomCu3	Grey, circular, shiny, smooth, raised	rod	G+**	Cu(II)
	RomCu4	Beige, circular, rough, raised	Oval shaped yeast cells, 3-4 μm		Cu(II)
	RomCu5	Beige, circular, rough, raised	Oval shaped yeast cells, 3-4 μm		Cu(II)
	RomCu6	White, circular, rough, raised	Oval shaped yeast cells, 3-4 μm		Cu(II)
	RomCu7	White, circular, glistering, raised	cocci	G+	Cu(II)
	RomCo1	Light-pink, circular, glistering, raised	rod	G+	-***
	RomCo4	Translucent, circular, glistering, raised	rod	G-	Co(II)
	RomHg1	White, circular, smooth, raised	Oval shaped yeast cells, 4-5 μm		-
	RomNi1	White, circular, glistering, raised	rod	G+	Ni(II)
	RomNi2	Beige, circular, glistering, raised	cocci	G+	Ni(II)
	RomNi3	Light-pink, circular, glistering, raised	rod	G+	Ni(II)
	RomNi4	White, circular, glistering, raised	cocci	G-	Ni(II)

(*continuation of Table 2*)

	Strain	Morphology of the colony	Morphology of the cells	Gram stain	Metal accumulation in biomass
Volcano Tungurahua, Ecuador	VulHg1	Translucent, circular, glistering, raised	rod	G+	-
	VulHg2	Red, circular, glistering, raised	rod	G+	-
	VulNi1	White, circular, glistering, raised	cocci	G-	-
	VulNi3	Translucent, circular, glistering, raised	cocci	G+	Ni(II)
	VulNi4	White, circular, glistering, raised	cocci	G+	-
	VulNi5	Peachy, circular, glistering, raised	cocci	G+	Ni(II)
	VulCr1	White, circular, glistering, raised	cocci	G-	-
	VulCr3	Peachy, circular, glistering, raised	rod	G+	-
	VulCr5	Pink, circular, glistering, raised	cocci	G-	-
	VulCu1	Yellow, circular, glistering, raised	cocci	G+	Cu(II)
	VulCu2	Light-brown, circular, glistering, raised	rod	G-	Cu(II)
	VulCu3	Yellow, circular, rough, raised	cocci	G-	-
	VulCu4	Pink, circular, glistering, raised	rod	G+	-
	VulCo1	White, circular, glistering, flat	cocci	G-	Co(II)
	VulCo2	White, circular, glistering, raised	rod	G-	-
	VulCo3	Pink, circular, glistering, raised	rod	G+	-

Note: * - gram negative bacteria;

** - gram positive bacteria;

*** - metal accumulation in biomass was not revealed by means of hydrogen sulphide contrasting: $Me^{2+} + H_2S = MeS\downarrow + 2H^+$

Sixteen strains were isolated from the ashes of volcano Tungurahua, only 5 of which could accumulate toxic metals (Table 2). The group of strains isolated out of batch cultures in the presence of 3000…10000 ppm of Cu^{2+} is of particular interest because of theirs high resistance to copper and ability to interact with it. The growth of one of these strains is presented on Fig. 14. It is a yeast strain, so as the majority of yeasts [5,8], it accumulates metals. The strain's biomass changed colour to dark-brown after contact with H_2S, which evidenced CuS formation in biomass and clearly Cu^{2+} accumulation.

The outcome of the study is that MC of Andes and ashes of the volcano Tungurahua are super resistant to representative toxic metals. The maximum permissible concentrations of toxic metals in 2-4 orders overweight bactericidal concentrations for most chemoorganotrophic microorganisms. Resistance of studied MC to ultra high concentrations of toxic metals, especially to copper, proves the thermodynamic prognosis of microbial interaction with toxic metals. According to the thermodynamic prognosis microorganisms are able to grow at very high concentrations of toxic metals.

The concentration succession was observed for studied MC. Dominate position was consistently occupied by bacteria, yeasts and filamentous fungi at Cu^{2+} concentration increasing. The "yeast link" was absent when concentration of other representative metals (Cr(VI), Hg^{2+}, Co^{2+}, Ni^{2+}) increased.

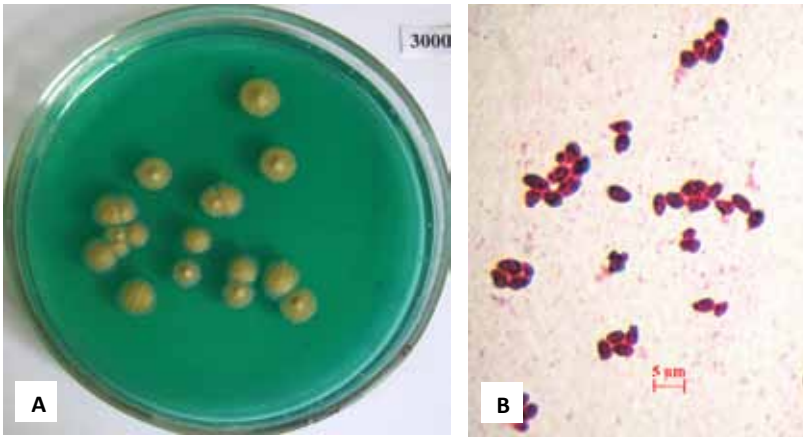

Fig. 14. Growth of yeast strain RomCu5 at 3000 ppm of Cu^{2+}; A – morphology of colonies; B – morphology of cells.

There were two types of response of microbial communities to metal concentration increasing. The first type of response is a catastrophic inhibiting of the growth microbial. The second type of response is the absence of microbial inhibition when metals concentrations increased.

Hydrogen was synthesized by microorganisms in the presence of toxic metals, which indicates diversified metal resistant microbial community. Synthesis of H_2 in the presence of toxic metals seems to be caused by anaerobic metal resistant microorganisms.

Metal resistant strains were isolated from the Andes cliff and volcanic ash ecosystems. Some of these strains accumulate toxic metals, and accordingly, extract metals from the environment. Such strains are promising for further development of biotechnologies for metal wastewater treatment from a wide range of toxic metals in any concentration range.

Таширев О.Б.[1], Прекрасна Є.П.[1], Таширева Г.О.[1], Белікова О.Ю.[2]

[1] Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України, вул. Академіка Заболотного, 154, Київ, МСП, Д03680, Україна
[2] Національний авіаційний університет, просп. Космонавта Комарова, 1, Київ, 03680, Україна

СТІЙКІСТЬ МІКРОБНИХ УГРУПОВАНЬ ВИСОКОГІРНИХ ЕКОСИСТЕМ ЕКВАДОРУ ДО РЕПРЕЗЕНТАТИВНИХ ТОКСИЧНИХ МЕТАЛІВ - CrO_4^{2-}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}

Резюме

Показано, що мікробні угруповання еквадорських Анд і попелу вулкану Тунгурауа є надстійкими до репрезентативних токсичних металів. Максимально допустимі концентрації токсичних металів складають 20 і 100 мг/л Hg^{2+}, 500 мг/л Co^{2+} і Ni^{2+}, 1000 і 1500 мг/л Cr(VI), 10000 і 20000 мг/л Cu^{2+}. Був вивчений вплив підвищення концентрації токсичних металів на приріст біомаси мікроорганізмів, синтез CO_2 та H_2. Виявлено два типи відповіді мікробних угруповань на підвищення концентрації токсичних металів. Перший тип відповіді – катастрофічне інгібування росту мікроорганізмів. Другий тип відповіді – відсутність інгібування росту мікроорганізмів при підвищенні концентрації токсичних металів. Вперше показана концентраційна сукцесія якісного складу мікробних угруповань Еквадору. При підвищенні концентрації Cu^{2+} домінуюче положення в мікробних угрупованнях послідовно займають бактерії, дріжджі, а потім мікроміцети. Вперше з екосистем кліфу еквадорських Анд і попелу вулкану Тунгурауа було виділено мікроорганізми, які є стійкими до надвисоких концентрацій токсичних металів (наприклад 3000...20000 мг/л Cu^{2+}) та накопичують токсичні метали.

Ключові слова**:** мікробні угруповання Еквадору, металрезистентність.

Таширев А.Б.[1], Прекрасная Е.П.[1], Таширева А.А.[1], Беликова Е.Ю.[2]

[1] Институт микробиологии и вирусологии им. Д.К. Заболотного НАН Украины, ул. Академика Заболотного, 154, Киев, МСП, Д03680, Украина
[2] Национальный авиационный университет, просп. Космонавта Комарова, 1, Киев, 03680, Украина

УСТОЙЧИВОСТЬ МИКРОБНЫХ СООБЩЕСТВ ВЫСОКОГОРНЫХ ЭКОСИСТЕМ ЭКВАДОРА К РЕПРЕЗЕНТАТИВНЫМ ТОКСИЧНЫМ МЕТАЛЛАМ - CrO_4^{2-}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}

Резюме

Показано, что микробные сообщества эквадорских Анд и пепла вулкана Тунгурауа являются сверхустойчивыми к репрезентативным токсичным металлам. Максимально допустимые концентрации токсичных металлов составляют 100 мг/л Hg^{2+}, 500 мг/л Co^{2+} и Ni^{2+}, 1000 и 1500 мг/л Cr(VI), 10000 и 20000 мг/л Cu^{2+}. Изучено влияние повышения концентрации токсичных металлов на прирост биомассы микроорганизмов, синтез CO_2 и H_2. Обнаружено два типа ответа микробных сообществ на повышение концентрации токсичных металлов. Первый тип ответа – катастрофическое ингибирование роста микроорганизмов. Второй тип ответа – отсутствие ингибирования роста микроорганизмов при повышении концентрации токсичных металлов. Впервые показана концентрационная сукцессия качественного состава микробных сообществ Эквадора. При повышении концентрации Cu^{2+} доминирующее положение в микробных сообществах последовательно занимают бактерии, дрожжи, а затем микромицеты. Впервые из экосистем клифа эквадорских Анд и вулканического пепла Тунгурауа были изолированы микроорганизмы, устойчивые к сверхвысоким концентрациям токсичных металлов (например, 3000...20000 мг/л Cu^{2+}) и способные накапливать токсичные металлы.

Ключевые слова: микробные сообщества Эквадора, металлрезистентность.

1. *Таширев А.Б.* Теоретические аспекты взаимодействия микроорганизмов с металлами. Восстановительная трансформация металлов. // Мікробіол. журнал. – 1994. – 56, № 6. – С. 76–88.
2. *Таширев А.Б.* Теоретические аспекты взаимодействия микроорганизмов с металлами. Микробная аккумуляция металлов, обусловленная их стереохимической аналогией с макроэлементами. // Мікробіол. журнал. – 1994. – 56, № 6. – С. 89–100.
3. *Таширев А.Б.* Взаимодействие микроорганизмов с металлами // Мікробіол. журн. – 1995. – 57, № 2. – С. 95–104.
4. *Таширева Г.О., Іутинська Г.О., Таширев О.Б.* Стійкість до іонів Cu^{2+} антарктичних штамів *Enterobacter hormaechei* ТА *Brevibacterium antarcticum* за різних умов культивування. // Мікробіол. журнал. – 2009. 71, № 4. – С. 3–9.
5. *Ховрычев М.П.* Поглощение ионов меди клетками *Candida utilis* // Микробиология. – 1973. – Т. XLII, № 5. – С. 839–843.
6. *Babich H., Stotzky G.* Nickel Toxicity to Microbes: Effect of pH and Implications for Acid Rain // Environmental research – 1982. – 29. – P. 335–350.
7. *Babich H., Stotzky G.* Differential toxicities of mercury to bacteria and bacteriophages in sea and in lake water // Can. J. Microbiol. – 1979. – 25, № 11. – P. 1252–1257.
8. *Blackwell K. J.*, *Singleton I., Tobin J. M* Metal cation uptake by yeast: a review Appl Microbiol Biotechnol – 1995. – 43. – P. 579–584.
9. *Chao W.H., Chen Cheryl L.F.* Role of exopolymer and acid-tolerance in the growth of bacteria in solutions with high copper ion concentration // L. Gen. Anot Appl. Microbiol. – 1991. – 37, № 4. – P. 363–370.
10. *Gupta S.L. Microcystis.* Interactive effects of nitrogen and copper on growth of cyanobacterium *Microcystis* // Bull. Environ. Contam. and Toxicol. – 1989. – 42, № 2. – P. 270–275.
11. *Furst P., Hu S., Hackett R., Hamer D.* Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein // Cell. – 1988. – 55, № 4. – P. 705–717.

12. *Ochoa-Herrera, V., León, G., Banihani, Q., Field, J.A., Sierra-Alvarez*. Toxicity of copper(II) ions to microorganisms in biological wastewater treatment systems //Science of the Total Environment. – 2011. – 412-413. – P. 380–385.
13. *Pourbaix M.* Atlas of electrochemical equilibria in aqueous solutions.—Oxford: Pergamon press. – 1963. – P. 320.
14. *Tashyrev O., Prekrasna Ie. Tashyreva G., Matvieieva N., Rokitko P., Romanovskaya V*. Regularities of metal resistant microorganisms` distribution in natural ecosystems // Екологично инженерство и опазване на околната среда. – 2013. – 2. – С. 39–50.
15. *Towiner S.B.* Copper sulfate helps control microorganisms in reservoirs // Water and Sewage Works. – 1976. – 123, N 12. – P. 68–70.
16. *Yamamoto H.R., Totsuyama K., Uchiwa,*. Fungal flora of soil polluted with copper. // Soil Biol. Biochem. – 1985. – 17. – P 785–790.
17. *Zibilski L.M., Wagnar G.H.,* Bacterial growth and fungal genera distribution in soil amended with sewage sluge containing cadmium, chromium and copper. // Soil. Sci. – 1982. – № 134. – P. 364–369.

Отримано 13.05.2014