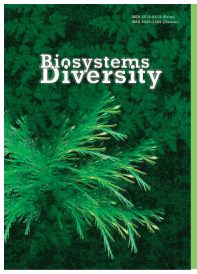

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Sulfidogenic and metal reducing activities of *Desulfuromonas* genus bacteria under the influence of copper chloride

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The selection of strains isolated from technogenically altered ecotopes and resistant to contamination, capable of metabolizing a wide range of pollutants is a task highly relevant for creation of new methods for environmental purification. Sulphur-reducing bacteria of the *Desulfuromonas* genus carry out dissimilatory reduction not only of S^0 but also oxidized forms of metals. Intensity of anaerobic respiration of microorganisms in polluted environments is determined by level of their adaptation to stress factors, in particular, copper (II) compounds. The aim of this work was to investigate the influence of copper (II) chloride on H_2S production by *Desulfuromonas* sp. strains isolated by us from Yavorivske Lake, to determine the efficiency of Cu^{2+} precipitation by hydrogen sulfide, to analyse the possibility of usage by bacteria of $CuCl_2$ as an electron acceptor of anaerobic respiration and to study the influence of Cu^{2+} on usage by these microorganisms of ferric (III) citrate, potassium dichromate or manganese (IV) oxide as electron acceptors. Bacteria were grown under anaerobic conditions in Kravtsov-Sorokin medium. To study the influence of Cu^{2+} on production by bacteria of H_2S, their cells were incubated with $CuCl_2$ (0.5–4.0 mM), washed and cultivated in a medium with S^0. To determine the level of Cu^{2+} binding by H_2S, produced by bacteria, cells were grown in a medium with $CuCl_2$ (0.5–4.0 mM) and S^0. To investigate the ability of bacteria to use copper (II) ions as electron acceptors, they were cultivated in a medium with $CuCl_2$ (1.74–10.41 mM). To study the influence of Cu^{2+} on usage by bacteria of metal compounds as electron acceptors, their cells were incubated with $CuCl_2$ (0.5–4.0 mM), washed and cultivated in media with $C_6H_5O_7Fe$, $K_2Cr_2O_7$ or MnO_2 (1.74–10.41 mM). Biomass was determined by the turbidimetric method. In the cultural liquid the content of H_2S was determined quantitatively by the spectrophotometric method, qualitatively – presence of Cu^{2+}. Content of CuS in the growth medium was determined by weight method. *Desulfuromonas* sp. bacteria was revealed to be resistant to 2.0–2.5 mM copper (II) ions. Under the influence of 3.0–4.0 mM $CuCl_2$ in the incubation mixture, sulfidogenic activity of bacteria decreased more than twice. The efficiency of Cu^{2+} binding in form of CuS by H_2S produced by bacteria reached 97.3–100.0% at presence in the medium with S^0 of up to 1.5 mM $CuCl_2$. Bacteria used $CuCl_2$ (1.74–10.41 mM) as an electron acceptor in the process of anaerobic respiration. The addition of 2.5–3.0 mM $CuCl_2$ to the incubation mixture caused inhibition of metal reducing activity of cells, growth of all strains in media with 1.74–10.41 mM ferric (III) citrate, potassium dichromate or manganese (IV) oxide as electron acceptors decreased by 2.6 times. Almost complete precipitation up to 1.5 mM copper (II) ions in form of CuS by H_2S produced by bacteria and ability to reduce up to 10.41 mM $CuCl_2$, $C_6H_5O_7Fe$, $K_2Cr_2O_7$ or MnO_2 in the process of anaerobic respiration indicates a high adaptation of the bacteria strains investigated by us to stress factors, in particular, the influence of $CuCl_2$. We have proved the possibility of using *Desulfuromonas* sp. in biotechnologies for purification of environments with complex contamination from copper (II) compounds.

Keywords: sulphur-reducing bacteria; hydrogen sulfide; copper; heavy metals.

Introduction

In nature, Cu (6.2×10^{-2} mass %) occurs both in a free state and in the form of compounds. Much copper is concentrated in the earth's crust in the form of sulfides ($CuFeS_2$ – chalcopyrite; Cu_2S – copper sulfide, chalcocite; CuS – covellite). Copper is part of oxides (Cu_2O – cuprite) and carbonates ($(CuOH)_2CO_3$ – malachite) (Betehtin, 2007). The total content of copper in the upper layers of soil varies within 5–50 mg/kg (Kucherjavyj, 2001). Excessive amounts of copper come into the environment from pesticides, in particular fungicides, sewage of galvanic plants. In the flooded quarries of sulphur deposits, waste water from industrial enterprises, waste heaps of coal mines, landfills of household and industrial waste, the content of Cu can grow by a thousand times (Frank & Lushnikov, 2006; Diakiv et al., 2016). In the technogenic reservoir which was formed in the place of the Yavoriv sulphur deposit quarry in recent years the content of copper (II) ions at depths of 0–70 m does not exceed 0.017–0.021 mg/l (Moroz et al., 2017).

The toxic effect of copper on cells of living organisms is due to its interaction with the SH-groups of amino acids of structural proteins and enzymes, which leads to their denaturation or inactivation (Ladomersky & Petris, 2015; Martínez-Bussenius et al., 2017). In the cases of chronic intoxication of humans with copper there can be frustrations of the nervous system, disorders of the kidneys and liver functions, dermatitis. Oxidized compounds of Cu, Hg and Cr are considered to be the most toxic to microorganisms, compared to other heavy metals (Tashirev et al., 2007; Cidre et al., 2017). Heavy metals pro-oxidants, such as Cu^{2+}, which are characterized by high oxidation-reduction potential (E_0'), are toxic and inhibit the growth of microorganisms (Tashirev et al., 2008; Richter et al., 2012; Viti et al., 2014). Due to their presence in the cell, the microbial metabolism changes in direction of metal reduction instead of electron acceptors with lower E_0' ($C_4H_4O_4$, SO_4^{2-}, $S_2O_3^{2-}$, SO_3^{2-}, S^0, HCO_3^-) reduction, which leads to generation of chemically active intermediates, oxygen radicals, energy exhaustion of cells and their death (Gescher & Kappler, 2012; Ladomersky & Petris, 2015; Simonte et al., 2017). Copper as a trace element at low concentrations is required by microorganisms for oxidation-reduction processes, stabilization of molecules in the case of electrostatic interactions, regulation of osmotic pressure, as a cofactor of metal proteins and some enzymes (Kumar et al., 2010). Copper influences the membrane Na^+/K^+ ATP-ases, the structure and functions of nucleic acids, and the synthesis of phospholipids. It is

present in the composition of enzymes and enzyme complexes (Cu, Zn-superoxide dismutase, lysiloxidase, doramine-β-hydroxylase, ascorbate oxidase, cytochrome *c*-oxidase, glutamyl transferase, hyponitrite reductase and nitrogen oxide reductase) (Lengeler et al., 2005). At high concentrations, copper represses α-ketoglutarate dehydrogenase, inhibits nitrate reductase, vitamin B_{12} synthesis, processes of photosynthesis, fermentation and respiration in microorganisms due to reduced content of cytochromes *b* and *c* (Segin et al., 2016; Martínez-Bussenius et al., 2017). *Cop* transport system of copper (chromosomal *cop*-operon) and its regulation in *Enterococcus hirae* has been described (Solioz & Stoyanov, 2003; Winkelmann, 2008). Copper resistance, which is determined by plasmid genes, is described in *Xanthomonas* (Behlau et al., 2017; Richard et al., 2017a; Gochez et al., 2018), *Cupriavidus* (Gillan et al., 2017), *Pseudomonas*, *Escherichia* and other genera of bacteria (Silver & Walderhaug, 1995; Richard et al., 2017b).

The selection of strains isolated from technogenically altered ecotopes and adapted to contamination, which are capable of metabolizing a wide spectrum of pollutants is a particularly relevant task in terms of establishing the mechanisms of their resistance (Limcharoensuk et al., 2015; Si et al., 2015; Dey et al., 2016) and creation of new methods for environmental purification (Iwahori et al., 2014; Mustapha & Halimoon, 2015). Sulfidogenic sulphur-reducing bacteria which reduce elemental or polysulfide forms of sulphur by action of sulphur reductase or polysulfide reductase, localized in cytoplasmic membrane and bound with hydrogenase by cytochromes or quinones (Hedderich et al., 1999), with the formation of hydrogen sulfide, attract attention of biotechnologists as potential agents of purification of environments contaminated by metal compounds, because as a result of interaction of H_2S with bivalent metal ions their insoluble sulfides are formed and thus they are removed from the natural cycle of elements (Diakiv et al., 2017; Kiran et al., 2017). Previously we have shown that bacteria strains of *Desulfuromonas* genus, isolated from Yavorivske Lake, have high metal reducing activity and may except sulphur using $C_6H_5O_7Fe$, $K_2Cr_2O_7$ or MnO_2 as electron acceptors in the process of anaerobic destruction of organic compounds (Moroz et al., 2014; Moroz et al., 2017). Metal reducing bacteria with involvement of metal reductases (multiheme *c*-type cytochromes) (Kozlova et al., 2008; Richter et al., 2012) enzymatically reduce Fe (III), Cr (VI), Mn (IV), U (VI), Tc (VII), Pd (II), V (V), Mo (VI), Cu (II) etc., which is one of the mechanisms of protection of cells from their toxic effects (Gescher & Kappler, 2012; Viti et al., 2014; Maslovska & Hnatush, 2015). The components of the Mtr (metal reducing) electron transfer respiratory chain containing the complex of *c*-type cytochromes are best studied today in bacteria of the *Shewanella* and *Geobacter* genera (Gescher & Kappler, 2012; Breuer et al., 2015; Simonte et al., 2017). Membrane-bound metal reductases (tetra- and decaheme c-type cytochromes) in *S. frigidimarina* are localized in the periplasm through which electrons from the cytoplasm from the carbon compounds metabolism reactions through the inner and outer membranes and periplasm are transmitted outside the cells, where in fact the soluble or insoluble metal compounds are reduced (Lengeler et al., 2005; Kozlova et al., 2008; Richter et al., 2012). The reduction of cations or oxygen-containing metal anions by metal reductases is carried out outside the cell, which is accompanied by release of a significant amount of electrons into the medium, therefore bacteria with exoelectrogenic properties are considered as possible anode biocatalysts in microbial fuel cells (Bilyy et al., 2014; Simonte et al., 2017).

Studies of the influence of Cu^{2+} on H_2S biogenesis by bacteria of *Desulfuromonas* sp. are important for development of effective and profitable biological methods of regulating their level in transformed biotopes. Investigations of the ability of sulphur-reducing bacteria to reduce Cu^{2+} are necessary for deepening understanding of mechanisms of their adaptation to existence in environments contaminated by copper (II) compounds. Studies of the influence of $CuCl_2$ on the ability of bacteria to reduce other metals will allow us to evaluate their resistance to copper (II) cations and the possibility of their application in environmental remediation technologies. Therefore, the purpose of this work was to investigate the influence of copper (II) chloride on H_2S production by sulphur-reducing bacteria of the *Desulfuromonas* genus isolated from Yavorivske Lake, to determine the efficiency of copper (II) cations precipitation by hydrogen sulfide, to analyse the possibility of usage by bacteria of $CuCl_2$ as an electron acceptor of anaerobic respiration and to study the influence of Cu^{2+} on usage by bacteria of ferric (III) citrate, potassium dichromate or manganese (IV) oxide as electron acceptors to assess the biotechnological potential of the investigated strains and to expand theoretical knowledge about changes in metabolism of sulphur-reducing bacteria under the influence of stress factors.

Materials and methods

Sulphur-reducing bacteria *Desulfuromonas acetoxidans* IMV B-7384, isolated by us earlier from Yavorivske Lake, are identified and stored at the Depository of D. K. Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine and in the collection of Microbiology Department of Ivan Franko National University of L'viv (Gudz et al., 2013). Bacteria *Desulfuromonas* sp. Yavor-5 and *Desulfuromonas* sp. Yavor-7, isolated by us from the same lake, are identified and stored in the collection of Microbiology Department of Ivan Franko National University of L'viv (Moroz et al., 2013).

The bacteria were grown in Kravtsov-Sorokin medium (Gudz et al., 2014) without Mohr's salt and without sulfate ions with sulphur of the following composition (g/l): $NaH_2PO_4 \times 12H_2O$ – 0.84, K_2HPO_4 – 0.5, NH_4Cl – 0.16, $MgCl_2 \times 6H_2O$ – 0.10, $NaC_3H_5O_3$ – 2.0. Before bacteria seeding, 0.05 ml of $Na_2S \times 9H_2O$ (1%) sterile solution was added to the medium. A sterile 10 N NaOH solution was used to provide pH of the medium to 7.2. Cells were added to the medium in quantity of 10 vol. % to initial concentration 10^8 CFU/ml (0.05 g/l). The sulphur was sterilized separately (0.5 atm) and placed in the medium at concentration of not less than 0.11 g/l (3.47 mM – concentration of SO_4^{2-} in medium of standard composition). Bacteria were grown for 10 days in tubes of 25 ml volume under anaerobic conditions at temperature of 30 °C.

Biomass was determined by the turbidimetric method using KFK-3 at 340 nm wavelength and 3 mm optical path and calculated in g/l using the formula: $C = (E_{340} \cdot n) / K$, where E – extinction at 340 nm wavelength, n – dilution, times, K – coefficient of recalculation, obtained by calibration curve of dependence of extinction from the mass of dry cells, determined by weight method and equal to 0.72 (Gudz et al., 2014). The concentration of hydrogen sulfide in the cultural liquid, separated from cells by centrifugation (4025 g, 20 min), was determined spectrophotometrically by the formation of methylene blue due to interaction of N,N-dimethyl-n-phenylenediamine dihydrochloride and hydrogen sulfide (Gudz et al., 2014).

To determine the influence of $CuCl_2$ on growth and hydrogen sulfide production by bacteria cells, previously grown in medium with fumarate ($C_4H_4O_4$) as electron acceptor (3.47 mM) and cysteine ($C_3H_7NO_2S$) at concentration of 0.2 g/l (Lengeler et al., 2005) to meet assimilation needs of bacteria in sulphur, were precipitated by centrifugation (4025 g, 20 min), resuspended in NaCl (0.9%) sterile solution and incubated for one hour with sterile solution of $CuCl_2$ at concentrations: 0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 mM, under sterile conditions. Cells were precipitated by centrifugation, washed twice with NaCl (0.9%) solution and sown in medium with S^0. After 10 days of growth the biomass and the content of hydrogen sulfide in the cultural liquid were determined.

The level of Cu^{2+} binding by H_2S, produced by bacteria, was determined. For this, bacteria were grown in a medium with S^0 and $CuCl_2$ at concentrations: 0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 mM. After 10 days of growth biomass was determined. The mixture of cells and CuS was precipitated by centrifugation (4025 g, 20 min), in the cultural liquid the presence of copper (II) cations was determined qualitatively (Harris, 2003) and hydrogen sulfide concentration quantitatively, the total concentration of which was calculated as sum of concentrations of free H_2S and bound in form of metal sulfide. The content of CuS was determined by weight method. For this, a mixture of cells and CuS was weighed; the mass of metal sulfide was calculated as difference between mass of mixture and dry cells (grown after incubation with $CuCl_2$) and medium components. The relative concentration (%) of bound by H_2S metal cations was calculated based on the ratio of molar concentrations of formed copper (II) sulfide and copper (II) ions, added into the medium at the beginning of bacteria cultivation, taking $CuCl_2$ concentration as 100%. To study the biomass accumulation by bacteria during usage of $CuCl_2$ or $C_4H_4O_4$ (control) as electron acceptors, cells

were sown in medium without S^0 with $C_3H_7NO_2S$ (0.2 g/l) as sulphur source. Sterile 1 M solutions of fumarate and copper (II) chloride were added into medium to their final concentrations that were 0.5, 1.0, 1.5, 2.0 and 3.0 times different from the standard electron acceptor content in Kravtsov-Sorokin medium (1.74, 3.47, 5.21, 6.94, 10.41 mM). Previously the bacteria were grown in a medium without S^0 with fumarate (3.47 mM) and cysteine (0.2 g/l) to the middle of the exponential growth phase. At 2, 4, 6, 8, 10 days biomass was determined. In the cultural liquid the presence of copper (II) cations was determined qualitatively (Harris, 2003).

To investigate the influence of $CuCl_2$ on use by bacteria of $C_6H_5O_7Fe$, $K_2Cr_2O_7$ or MnO_2 as electron acceptors the cells, previously grown in a medium with $C_4H_4O_4$ (3.47 mM) and $C_3H_7NO_2S$ (0.2 g/l) to the middle of exponential growth phase, were precipitated by centrifugation (4025 g, 20 min), resuspended in sterile NaCl (0.9%) solution, under sterile conditions incubated for one hour with sterile $CuCl_2$ solution at concentrations: 0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 mM, precipitated by centrifugation, washed twice with NaCl (0.9%) solution and sown in a medium without S^0 with $C_3H_7NO_2S$ (0.2 g/l), to which added sterile solutions of $C_6H_5O_7Fe$, $C_4H_4O_4$ (control), $K_2Cr_2O_7$ or weighted quantities of insoluble in water MnO_2 to final concentrations 1.74, 3.47, 5.21, 6.94, 10.41 mM. $Na_3C_6H_5O_7$ was added to the medium with $C_6H_5O_7Fe$ instead of $NaC_3H_5O_3$. At day 10, biomass was determined.

Experiments were repeated three times with three parallel formulations for each variant of experimental and control conditions. The obtained data were processed by generally accepted methods of variation statistics. The reliability of the difference was evaluated using ANOVA. Differences between the samples were considered reliable at $P < 0.05$.

Results

To study the influence of Cu^{2+} on sulfidogenic activity of *Desulfuromonas* sp. the cells of bacteria, previously grown in medium with fumarate, were incubated for 1 hour with 0.5–4.0 mM $CuCl_2$, washed and cultivated in medium with S^0 (Fig. 1).

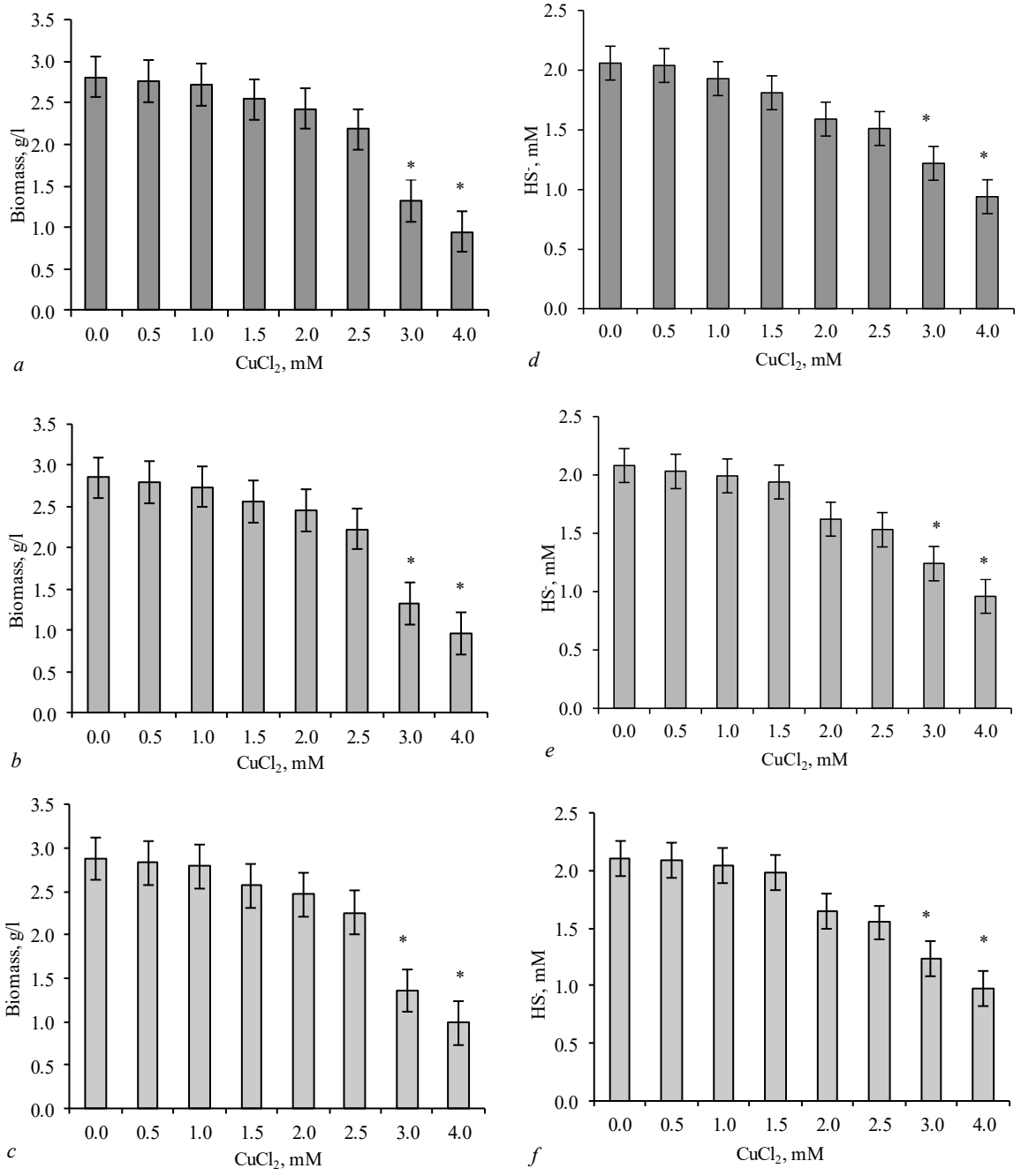

Fig. 1. The influence of $CuCl_2$ on biomass accumulation (*a*, *b*, *c*) and hydrogen sulfide production (*d*, *e*, *f*) by *D. acetoxidans* IMV B-7384 (*a*, *d*), *Desulfuromonas* sp. Yavor-5 (*b*, *e*) and *Desulfuromonas* sp. Yavor-7 (*c*, *f*) after 10 days of growth in medium with S^0; * – $P < 0.05$

To study the level of copper (II) ions binding by produced by bacteria, hydrogen sulfide cells were grown in medium with S^0 and $CuCl_2$ at different concentrations. Strains of bacteria of *Desulfuromonas* genus proved to be resistant to $CuCl_2$ influence at concentrations of 0.5–2.5 mM both in the incubation mixture and in the cultivation medium. Under the influence of 3.0–4.0 mM $CuCl_2$, the accumulation of biomass and the production of H_2S by sulphur-reducing bacteria in the medium with elemental sulphur decreased by 2.1–3.0 and 1.7–2.2 times, respectively (Fig. 1).

The cells of bacteria, incubated with 0.5–1.5 mM $CuCl_2$, in 10 days produced not less than 1.8–2.0 mM H_2S. Therefore, the efficiency of copper (II) ions binding in form of CuS by hydrogen sulfide produced by bacteria reached 97.3–100.0% at the presence in medium with S^0 of 0.5–1.5 mM $CuCl_2$ (Table 1). After addition during seeding into the medium of up to 1.5 mM copper (II) cations for 10 days they were completely binded with the H_2S, produced by bacteria, which confirmed the negative results of qualitative reactions on their presence in the cultural liquid. The binding level of copper (II) ions, added to medium at the beginning of cultivation at concentration of 2.0 mM, with the H_2S produced by bacteria was 1.3 times lower than at lower concentrations of $CuCl_2$ in the medium and did not exceed 81.0%, since it amount was not sufficient for full interaction with metal ions. After addition of more than 2.0 mM Cu^{2+} into the medium, positive results of qualitative reactions indicated the presence of metal ions in the cultural liquid.

Consequently, it was found that under the influence of 3.0–4.0 mM $CuCl_2$, sulfidogenic activity of *Desulfuromonas* sp. decreased more than twice. The efficiency of copper (II) ions precipitation in form of CuS by H_2S produced by bacteria reached 97.3–100.0% at presence in the medium with S^0 up to 1.5 mM $CuCl_2$. The influence of $CuCl_2$ on biomass accumulation by sulphur-reducing bacteria was studied. Cells were grown in media with cysteine as a source of sulphur and $CuCl_2$ or $C_4H_4O_4$ (control) at concentrations of 1.74, 3.47, 5.21, 6.94, 10.41 mM (Fig. 2).

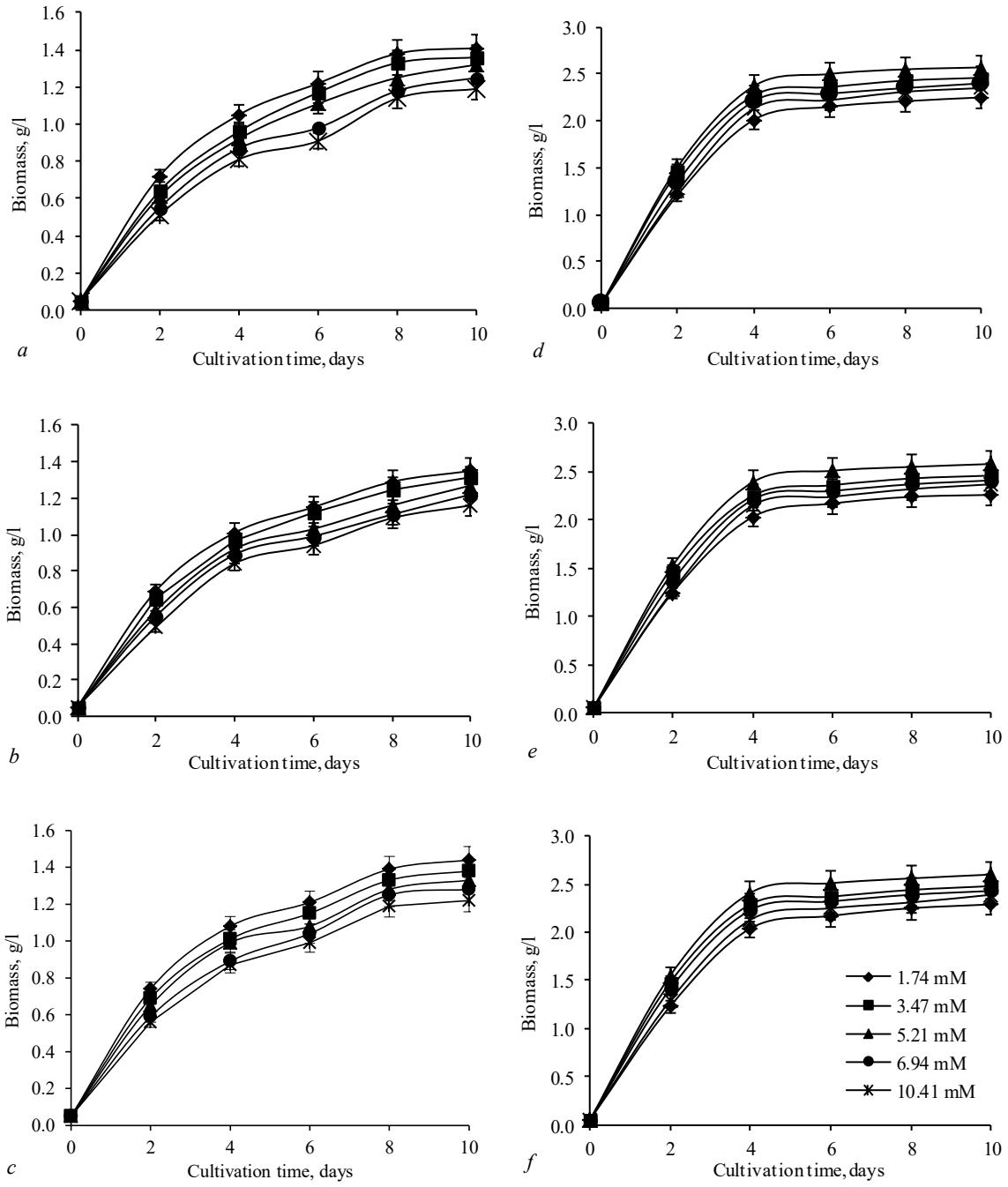

Fig. 2. Biomass accumulation by *D. acetoxidans* IMV B-7384 (*a*, *d*), *Desulfuromonas* sp. Yavor-5 (*b*, *e*) and *Desulfuromonas* sp. Yavor-7 (*c*, *f*) during growth in medium with $CuCl_2$ (*a*, *b*, *c*) or $C_4H_4O_4$ (*d*, *e*, *f*) at different concentrations

With increasing of $CuCl_2$ concentrations in the cultivation medium, the biomass of *Desulfuromonas* sp. strains decreased. The largest biomass of bacteria was accumulated for 8–10 days in the medium with the lowest concentration of copper (II) ions – 1.74 mM (up to 1.44 g/l), and the smallest – with the highest – 10.41 mM (up to 1.22 g/l), which can be explained by the toxic effect of metal ions on bacteria cells. At presence in the medium of $CuCl_2$ in all concentrations for 10 days, biomass of bacteria was 1.5–2.0 times lower than in the medium with fumarate. In contrast to the bacteria growth in the medium with $CuCl_2$, with increasing concentrations of fumarate in the cultivation medium from 1.74 to 3.47 mM for 4–10 days a rise in biomass accumulation by bacteria of all strains was observed from 2.29 to 2.48 g/l. However, the highest biomass (up to 2.60 g/l) bacteria accumulated in the medium with fumarate at concentration of 5.21 mM. With further growth of fumarate concentrations in the medium from 6.94 to 10.41 mM, the biomass decreased and did not exceed 2.39 g/l, possibly due to limiting of bacteria growth by other factors in the medium. Copper (II) ions were found in the medium throughout all time of bacteria cultivation (Table 2), which indicates its incomplete use, apparently, due to high toxicity.

Thus, it has been found that bacteria *Desulfuromonas* sp. used copper (II) ions as electron acceptors in the process of anaerobic respiration at concentrations of 1.74–10.41 mM $CuCl_2$ in the medium. A 1.5–2.0 times lower biomass yield was found during use by bacteria of copper (II) chloride, compared with use of fumarate in all investigated concentrations of electron acceptors in the medium. The highest biomass (up to 1.44 g/l) bacteria accumulated in the medium with 1.74 mM $CuCl_2$. For 10 days of cultivation, bacteria of all strains didn't completely reduce the copper (II) ions present in the medium.

To study the influence of Cu^{2+} on metal reducing activity of *Desulfuromonas* sp., bacteria cells, previously grown in a medium with fumarate were incubated for 1 hour with 0.5–4.0 mM $CuCl_2$, washed and cultivated in media with cysteine as sulphur source and $C_6H_5O_7Fe$, $C_4H_4O_4$ (control), $K_2Cr_2O_7$ or MnO_2 at concentrations of 1.74, 3.47, 5.21, 6.94, 10.41 mM. It has been established that with increasing concentrations of ferrum (III), chromium (VI) or manganese (IV) compounds in the medium, biomass accumulation by *Desulfuromonas* sp. non-incubated and incubated with $CuCl_2$ cells after 10 days of growth decreased (Fig. 3, 4).

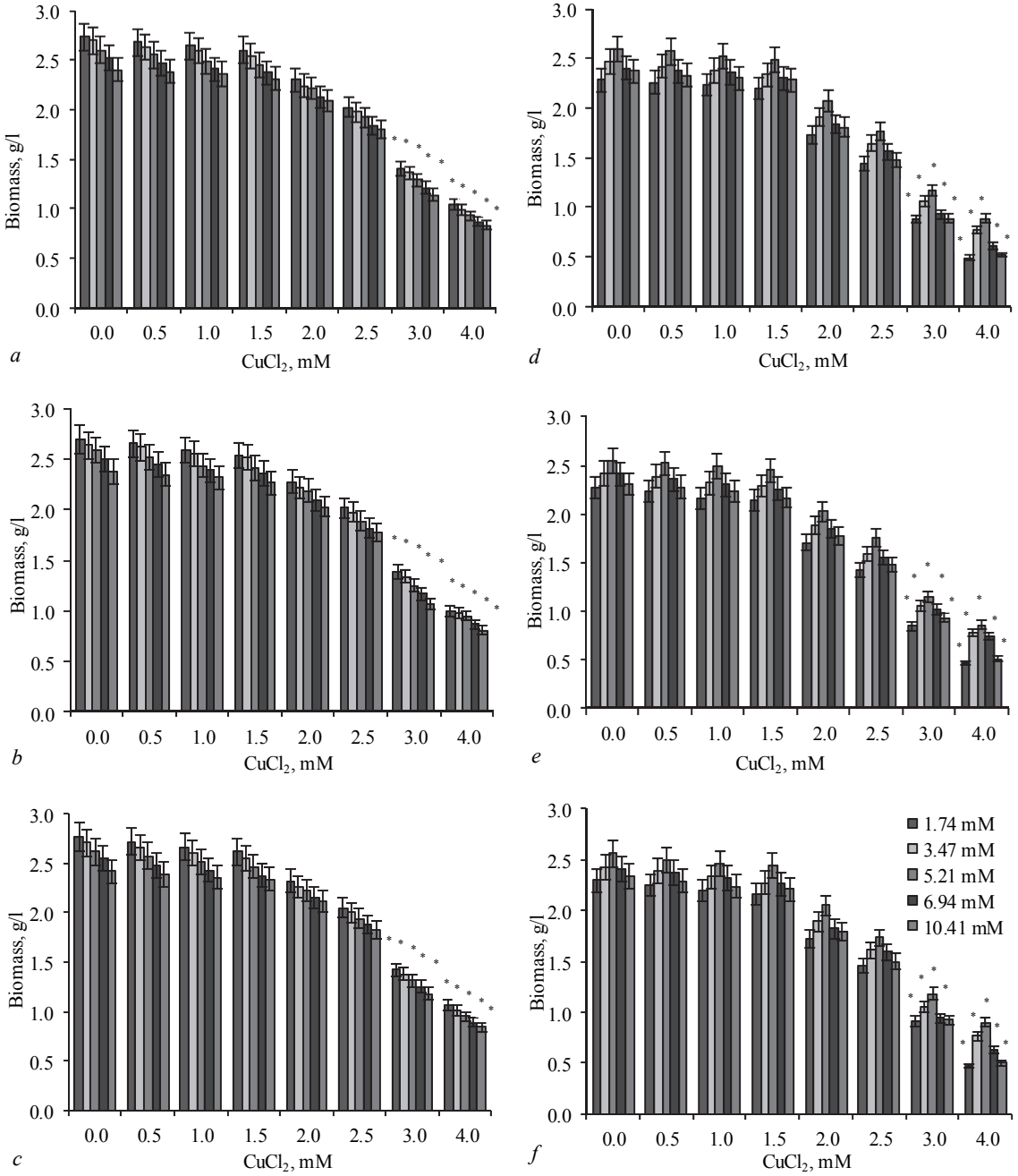

Fig. 3. The influence of $CuCl_2$ on biomass accumulation by *D. acetoxidans* IMV B-7384 (*a*, *d*), *Desulfuromonas* sp. Yavor-5 (*b*, *e*) and *Desulfuromonas* sp. Yavor-7 (*c*, *f*) after 10 days of growth in medium with $C_6H_5O_7Fe$ (*a*, *b*, *c*) or $C_4H_4O_4$ (*d*, *e*, *f*): * – $P < 0.05$

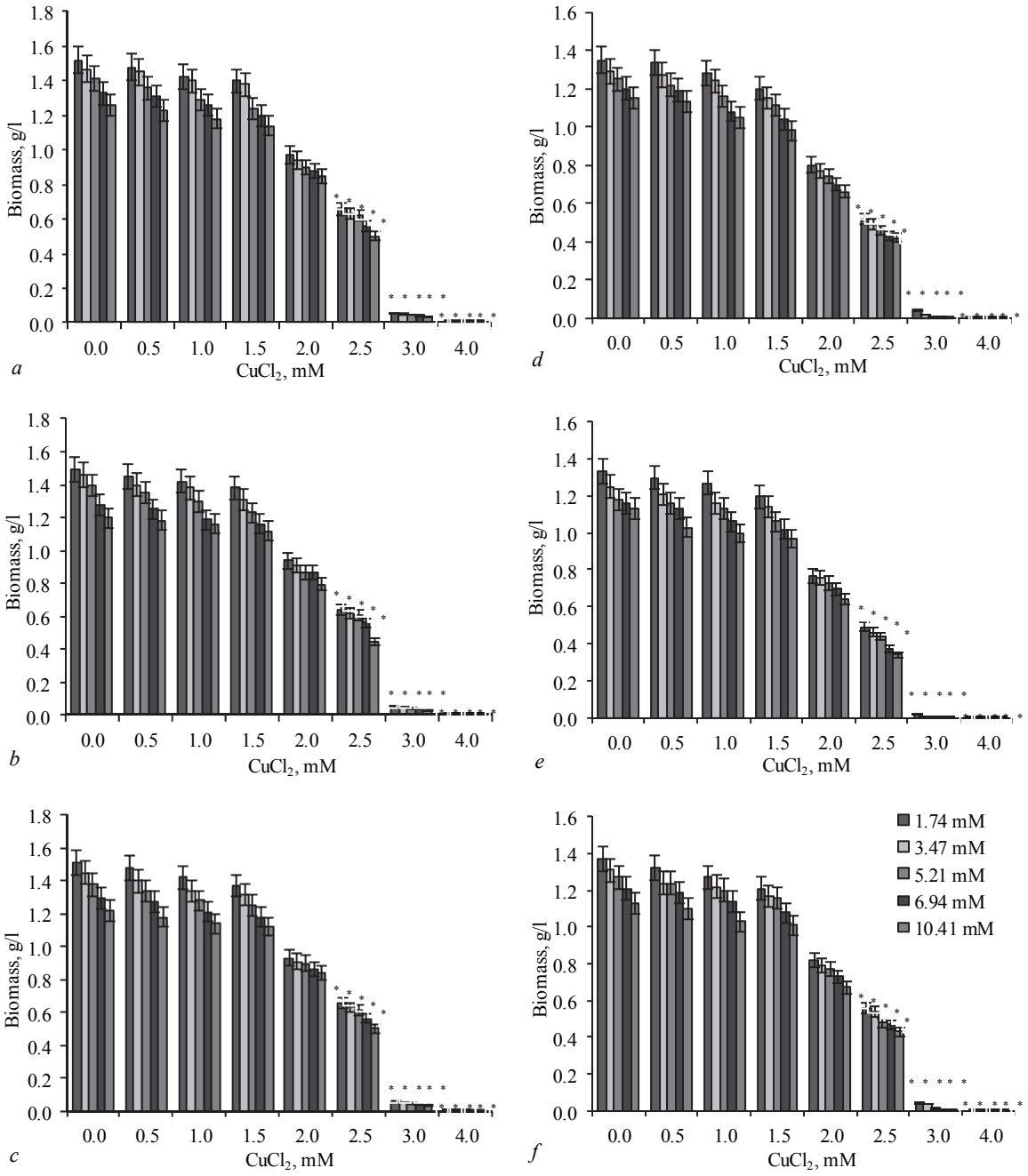

Fig. 4. The influence of $CuCl_2$ on biomass accumulation by *D. acetoxidans* IMV B-7384 (*a*, *d*), *Desulfuromonas* sp. Yavor-5 (*b*, *e*) and *Desulfuromonas* sp. Yavor-7 (*c*, *f*) after 10 days of growth in medium with $K_2Cr_2O_7$ (*a*, *b*, *c*) or MnO_2 (*d*, *e*, *f*): * – $P < 0.05$

In contrast to the growth of bacteria in media with metal compounds, with increasing of fumarate concentrations in the cultivation medium 1.74–5.21 mM, a rise in biomass accumulation by bacteria of all strains was observed, which decreased with further growth of its concentrations up to 10.41 mM. The bacteria cells, not incubated with $CuCl_2$, for 10 days accumulated the highest biomass in the medium with ferric (III) citrate (up to 2.77 g/l), in comparison with growth in media with fumarate (up to 2.60 g/l), potassium dichromate (up to 1.52 g/l) or manganese (IV) oxide (up to 1.37 g/l). With increasing concentrations of $CuCl_2$ during incubation, the biomass accumulation by bacteria in media with all tested electron acceptors decreased, which can be explained by the toxic effect of copper (II) ions on bacteria cells. However, copper (II) chloride at concentrations of 0.5–2.5 mM in the incubation mixture didn't have a significant negative effect on bacteria growth in media, both with fumarate and with metal compounds at all concentrations. Under the influence of 3.0–4.0 mM $CuCl_2$, the biomass of bacteria in media with 1.74–10.41 mM $C_6H_5O_7Fe$ and $C_4H_4O_4$ decreased by 2.1–2.9 and 2.6–4.9 times, respectively (Fig. 3). Under the influence of 2.5 mM $CuCl_2$, the biomass accumulation by bacteria in media with 1.74–10.41 mM $K_2Cr_2O_7$ and MnO_2 decreased by 2.4 and 2.6 times respectively, and incubated with 3.0–4.0 mM $CuCl_2$ cells of all strains in media with these electron acceptors practically didn't grow, probably due to the negative effect on them of potassium dichromate or manganese (IV) oxide and Cu^{2+} (Fig. 4).

Thus, it was found that metal reducing activity of sulphur-reducing bacteria cells, incubated with 0.5–2.0 mM $CuCl_2$, remained almost unchanged compared to activity of non-incubated cells, which demonstrates the high potential of application of these microorganisms in biotechnologies for purification of environments with complex pollution from heavy metals. Copper (II) chloride at concentrations of 2.5–3.0 mM in the incubation mixture inhibited growth of all *Desulfuromonas* sp. strains in media with $C_6H_5O_7Fe$, $K_2Cr_2O_7$ or MnO_2 as electron acceptors at concentrations 1.74–10.41 mM.

Discussion

Hydrogen sulfide, produced by sulfidogenic microbiota, interacts with heavy metal ions, precipitating them in form of sulfides (White

et al., 2000; Kiran et al., 2017). The formation of metal sulpfides is the main way of eliminating heavy metals from the natural cycle (White et al., 2000; Kozlova et al., 2008; Kuznetsov et al., 2015). The efficiency of microbiological precipitation of metal ions by hydrogen sulfide, produced by cells of sulphur-reducing bacteria, depends on concentration of H_2S, which they produced in process of dissimilatory sulphur reduction (Gudz et al., 2011). On the other hand, the intensity of anaerobic respiration in these microorganisms in contaminated ecotopes is determined by level of their adaptation to unfavourable environmental conditions, in particular, increased content of metal compounds (Viti et al., 2014; Si et al., 2015; Simonte et al., 2017). Among the bacteria of the *Desulfuromonas* genus, no resistance to copper species, or those that carry out its reduction, are described (Vandieken et al., 2006).

Table 1
Copper (II) sulfide formation in sulphur-reducing bacteria cultivation medium after addition S^0 and $CuCl_2$

Strain	$CuCl_2$, mM	CuS, mM	Copper (II) ions binding, %	Qualitative analysis on ions presence
D. acetoxidans IMV B-7384	control	0	0	–
	0.5	0.50 ± 0.05	100.0 ± 0.2	–
	1.0	0.98 ± 0.02	98.0 ± 0.3	–
	1.5	1.46 ± 0.01	97.3 ± 0.1	–
	2.0	1.58 ± 0.04	79.0 ± 0.4	+
	2.5	1.50 ± 0.03	60.0 ± 0.5	+
	3.0	1.19 ± 0.01	39.7 ± 0.1	+
	4.0	0.94 ± 0.02	23.5 ± 0.2	+
Desulfuromonas sp. Yavor-5	control	0	0	–
	0.5	0.50 ± 0.03	100.0 ± 0.1	–
	1.0	1.00 ± 0.01	100.0 ± 0.4	–
	1.5	1.47 ± 0.03	98.0 ± 0.3	–
	2.0	1.60 ± 0.02	80.0 ± 0.5	+
	2.5	1.52 ± 0.04	60.8 ± 0.1	+
	3.0	1.21 ± 0.05	40.3 ± 0.2	+
	4.0	0.95 ± 0.01	23.8 ± 0.1	+
Desulfuromonas sp. Yavor-7	control	0	0	–
	0.5	0.50 ± 0.02	100.0 ± 0.1	–
	1.0	1.00 ± 0.03	100.0 ± 0.4	–
	1.5	1.49 ± 0.04	99.3 ± 0.3	–
	2.0	1.62 ± 0.02	81.0 ± 0.2	+
	2.5	1.53 ± 0.02	61.2 ± 0.1	+
	3.0	1.22 ± 0.01	40.7 ± 0.4	+
	4.0	0.96 ± 0.03	24.0 ± 0.5	+

Note: "+" – copper (II) ions are present; "–" – copper (II) ions are absent.

To study the influence of copper (II) ions on sulfidogenic activity of sulphur-reducing bacteria, cells were incubated for one hour with $CuCl_2$ at different concentrations, washed and cultivated in a medium with elemental sulphur to avoid, on the one hand, extracellular reduction of Cu^{2+}, and, on the other, formation in the cultural liquid of insoluble metal sulfide. Often, it takes just five minutes to completely absorb metals with bacteria, but this time can range from a few seconds to an hour after contact with toxicant (White et al., 2000; Tashirev et al., 1995). It was assumed that the inhibition of metabolic processes in bacteria incubated with copper (II) ions would be due to their accumulation by surface structures (as result of binding with polysaccharides, outer membrane proteins, cell wall structures) or inside cells, where they may not inversely interact with structural components of cytoplasmic membrane, cytoplasm proteins and other metabolites (Lengeler et al., 2005; Tashirev et al., 2008). To determine the level of copper (II) ions precipitation by hydrogen sulfide produced by bacteria, cells were cultivated in a medium with S^0, to which $CuCl_2$ was added at different concentrations. Despite that, in case of simultaneous presence in medium S^0 and oxidized form of copper, bacteria will use (as assumed) $CuCl_2$ as the more energy efficient electron acceptor (oxidation-reduction potential of pair Cu^{2+}/Cu^+ $E_0' = +0.15$ V is higher than that of pair S^0/H^{S-} $E_0' = -0.27$ V) (Sani et al., 2001; Lengeler et al., 2005; Richter et al., 2012), at high concentrations it showed a more or less pronounced toxic effect on the microorganisms.

Previously, we found that bacteria of the *Desulfuromonas* genus, isolated from reservoir of the Yavoriv sulphur deposit, in process of anaerobic destruction of organic compounds with different intensity use up to 10 mM $C_6H_5O_7Fe$, $K_2Cr_2O_7$ and MnO_2 as final electron acceptors (Moroz et al., 2014, 2017). Therefore we studied the ability of these bacteria to reduce copper (II) cations in the process of anaerobic respiration. Whereas reduction of metals pro-oxidants by membrane-bound metal reductases is carried out outside the cell (Gescher & Kappler, 2012; Richter et al., 2012; Simonte et al., 2017), with increasing concentration of soluble metal compounds in the medium, the degree of their cations or oxoanions penetration through bacteria cytoplasmic membrane in cytoplasm increases, where their interaction with intracellular metabolites occurs, chemically active intermediates, oxygen radicals are formed, reduced forms of metals as end products are accumulated, which causes the inhibitory effect on growth (Richter et al., 2012; Viti et al., 2014).

Table 2
Cu^{2+} reducing by sulphur-reducing bacteria after addition into medium $CuCl_2$

Strain	Cultivation time, days	Cu^{2+}, mM				
		1.74	3.47	5.21	6.94	10.41
D. acetoxidans IMV B-7384	0	+	+	+	+	+
	2	+	+	+	+	+
	4	+	+	+	+	+
	6	+	+	+	+	+
	8	+	+	+	+	+
	10	+	+	+	+	+
Desulfuromonas sp. Yavor-5	0	+	+	+	+	+
	2	+	+	+	+	+
	4	+	+	+	+	+
	6	+	+	+	+	+
	8	+	+	+	+	+
	10	+	+	+	+	+
Desulfuromonas sp. Yavor-7	0	+	+	+	+	+
	2	+	+	+	+	+
	4	+	+	+	+	+
	6	+	+	+	+	+
	8	+	+	+	+	+
	10	+	+	+	+	+

Note: "+" – copper (II) ions are present; "–" – copper (II) ions are absent.

The efficiency of biological methods for purifying the environment from pollutants depends not only on metabolic activity of selected strains of bacteria, but primarily on their resistance to metal ions. Therefore, the influence of $CuCl_2$ at concentrations of 0.5, 1.0, 1.5, 2.0, 3.0 times different from the sulphur content in Kravtsov-Sorokin medium of standard composition on biomass accumulation by sulphur-reducing bacteria was investigated. It is known that bacteria reduce fumarate to succinate in the process of fumarate respiration with participation of electron transport chain, which contains a number of dehydrogenases and fumarate reductase, which are interconnected by pool of *b*-type cytochromes and menaquinones (Lengeler et al., 2005). In a medium with $C_4H_4O_4$, H_2S toxic for cells does not form and biomass yield is almost the same as in the medium with S^0 (Moroz et al., 2017), so a medium with fumarate was used as control to compare the biomass accumulation by bacteria during usage of copper (II) chloride or other metals compounds and $C_4H_4O_4$ as electron acceptors. Although oxidation-reduction potential of the pair Cu^{2+}/Cu^+ ($E_0' = +0.15$ V) is higher than that of the pair fumarate/succinate ($E_0' = +0.03$ V) (Lengeler et al., 2005), the use of $CuCl_2$ by microorganisms was very slow.

To study the influence of copper (II) ions on metal reducing activity of bacteria, the cells were incubated for one hour with $CuCl_2$ at different concentrations, washed (to avoid extracellular reduction of Cu^{2+}) and cultivated in media with ferric (III) citrate, potassium dichromate, manganese (IV) oxide or fumarate (control) at concentrations 1.74–10.41 mM. With increasing $CuCl_2$ concentrations during incubation, the growth of bacteria in media with all electron acceptors decreased, possibly due to its toxic effect on bacteria cells. Although oxidation-reduction potential of the pair $Cr_2O_7^{2-}/Cr^{3+}$ ($E_0' = +1.33$ V) is higher than that of the pairs Mn (IV)/Mn (II) ($E_0' = +1.23$ V) and Fe (III)/Fe (II) ($E_0' = +0.77$ V) and much higher than that of the pair fumarate/succinate ($E_0' = +0.03$ V)

(Lengeler et al., 2005), use of potassium dichromate and manganese (IV) oxide by microorganisms did not provide the high biomass accumulation.

Selection of strains of microorganisms from anthropogenically altered biotopes, which are characterized by increased content of sulphur and metal compounds, deserves special attention. Sulphur-reducing bacteria *D. acetoxidans* IMV B-7384, *Desulfuromonas* sp. Yavor-5 and *Desulfuromonas* sp. Yavor-7, isolated by us from Yavorivske Lake, were revealed to be highly resistant to copper (II) ions at concentrations much higher than those in this technogenic reservoir. Studies of their sulphur- and metal-reducing activities under the influence of stress factors, in particular action of $CuCl_2$ and compounds of other metals, is especially important, since formation of metal sulfides is the main way by which heavy metals are removed from the natural cycle (White et al., 2000; Kuznetsov et al., 2015; Kiran et al., 2017), and reduction of transitional heavy metal compounds by microorganisms in media with low oxidation-reduction potential plays an important role in the process of organic substrates oxidation (Kozlova et al., 2008; Gescher & Kappler, 2012; Simonte et al., 2017). Almost complete precipitation up to 1.5 mM of copper (II) ions in form of CuS by H_2S produced by bacteria and ability to reduce up to 10.41 mM $CuCl_2$, $C_6H_5O_7Fe$, $K_2Cr_2O_7$ or MnO_2 in the process of anaerobic respiration indicates a high adaptation of the bacteria strains investigated by us to stress factors, in particular, the influence of copper (II) chloride.

Investigation of mechanisms of interaction of sulfidogenic representatives of microbiota from Yavorivske Lake with heavy metals pro-oxidants which are highly toxic to microorganisms, such as Cu^{2+}, are necessary for the widest possible disclosure of their potential in transformation of sulphur, carbon and metal compounds and creation on their basis of new methods for protecting the environment from hazardous chemical pollutants.

Conclusions

Thus, it was established that the sulfidogenic activity of bacteria strains *Desulfuromonas* sp., isolated from Yavorivske Lake, under the influence of 0.5–2.5 mM $CuCl_2$ practically did not change. The efficiency of copper (II) ions precipitation in the form of CuS by hydrogen sulfide produced by bacteria reached 100.0% after addition into a medium with sulphur of up to 1.5 mM $CuCl_2$. Bacteria used copper (II) ions as an electron acceptor in the process of anaerobic respiration at concentrations of 1.74–10.41 mM in a medium with their incomplete reduction for 10 days of cultivation. The copper (II) chloride at concentrations of 0.5–2.0 mM in the incubation mixture did not significantly affect the biomass accumulation by bacteria in media with 1.74–10.41 mM $C_6H_5O_7Fe$, $K_2Cr_2O_7$ or MnO_2 as electron acceptors, which demonstrates the high application potential of these microorganisms in biotechnologies for purifying environments with complex pollution from heavy metals.

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