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# THE EFFECT OF SOME ESSENTIAL ENVIRONMENTAL FACTORS ON THE MICROBIAL DISSIMILATORY SULPHATE REDUCTION

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## ABSTRACT

The effect of some heavy metals (manganese, iron, uranium) and arsenic on microbial dissimilatory sulphate reduction was studied under laboratory conditions. It was found that the sulphate-reducing bacteria were able to grow at concentrations of $Mn^{2+}$, $As^{5+}$ and $U^{6+}$ as high as 50 mg/l but the rate of sulphate reduction was decreased by about 15 – 55 % in the different experiments. The rate of sulphate reduction, however, was increased in the presence of high concentration of $Fe^{2+}$ (in the range of 0,5 – 2,0 g/l). The sulphate-reducing bacteria efficiently precipitated the above-mentioned agents, as well as the ferrous iron. The microbial sulphate reduction was carried out at pH values higher that 4,9. The bacteria were able to increase the pH of the solutions to neutral or slightly alkaline level by the hydrocarbonate ions generated during the dissimilatory sulphate reduction. In the presence of both nitrate and sulphate ions, the microbial dissimilatory nitrate reduction was always the prevalent process. The concentrations of nitrate as high as 500 mg/l inhibited strongly the rate of sulphate reduction.

## INTRODUCTION

Bacterial sulphate reduction has been indentified as a significant mechanism for removing contaminant heavy metals, toxic and radioactive elements from coal and metal-mine drainage (*Gazea et al., 1995; James et al., 1995*). Sulphate-reducing bacteria oxidaze simple organic compounds (such as lactate, acetate, butirate and another products of fermentations) with sulphate under anaerobic conditions, and thereby generate hydrogen sulphide and bicarbonate ions:

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- , \qquad (1)$$

where $CH_2O$ represent the organic matter.

The produced hydrogen sulphide reacts with dissolved metals to form insoluble metal sulphides that subsequently precipitate according to the reaction:

$$M^{2+} + H_2S \rightarrow MS + 2H^+, \qquad (2)$$

where M includes metals such as Fe, Cu, Zn, Ni, Cd.

The hydrogen sulphide can chemically reduce $As^{5+}$ to $As^{3+}$, resulting in the formation of minerals such as orpiment ($As_2S_3$) and/or an arsenic-iron sulphide solid phase (*Ronalds et al., 2000*). Some of sulphate-reducing bacteria formed orpiment in culture by mediating biological reduction of $As^{5+}$ followed by the reduction of sulphate during growth.

Certain sulphate-reducing bacteria can enzymatically reduce also highly soluble hexavalent uranium to the sparingly soluble tetravalent form $U^{4+}$. Reduced uranium then abiotically precipitates as uraninite ($UO_2$) (*Ganesh et al., 1997*). The reduction of $U^{6+}$ can carried out by a bacterial produced hydrogen sulphide in the presence of high concentrations of both U and sulphide and/or elevated temperatures (*Barnes et al., 1993*) according to the following reaction:

$$4[UO_2(CO_3)_3]^{4-} + HS^- + 15H^+ \rightarrow 4UO_2 + SO_4^{2-} + 12CO_2 + 8H_2O \qquad (3)$$

Although at low pH the growth and activity of sulphate-reducing bacteria are inhibited, their activity results in pH increments in their immediate environment, allowing their viability in the solid organic substrate, independing of drainage extremely acidic waters. Bicarbonate ions formed during sulphate reduction react with protons to form $CO_2$ and water and remote acidity from solutions as $CO_2$ gas:

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O \qquad (4)$$

The bicarbonate ions buffer the waters pH to a particular value, typically in the range of 6 – 7 (*Darryl et al., 1992*). Raising the pH of acidic water will couse some metals to hydrolyze and precipitate as insoluble hydroxides or oxides; for example,

$$Al^{3+} + 3H_2O \rightarrow Al(OH)_3 + 3H^+ \qquad (5)$$

The process of microbial sulphate reduction has been found a large application in treatment of acid mine waters by SRB-bioreactors, reactive barriers and passive systems. The pollutants are retained in strict anaerobic conditions by used of waste organic mater (a mixture of cow, horse and sheep manure, spent mushroom compost and sawdust) or chip row materials (acetate, ethanol, est.) as sources of carbon and energy by sulphate-reducing bacteria.

The purpose of this study was to evaluate the effect of heavy metals Mn, U, Fe and arsenic on the growth and activity of sulphate-reducing bacteria; the efficiency of precipitation of above-mentioned toxic elements; to find the lowest pH value, in which the bacterial sulphate-reduction is possible; to evaluate the effect of high concentrations nitrate on the rate of the process.

## MATERIALS AND METHODS

Batch experiments were carried out in 500 ml glasses bottles containing organic substrates and nutrient solution. Simple organic compounds (Na-lactate, Na-acetate, Na-propionate, glycerol) or solid organic matter (cow manure, spent mushroom compost and sawdust) were used as organic substrates. The bottles were inoculated with mixed enrichment culture of sulphate-reducing bacteria belong to p.Desulfotomaculum, p.Desulfovibrio and p.Desulfobacter.

### The effect of U, As, Mn and Fe on dissimilatory sulphate reduction

The nutrient solution used in these tests contained (in g/l): $Na_2SO_4$, 3.0; $KH_2PO_4$, 0.2; $(NH_4)_2SO_4$, 0.5; KCl, 0.5; $CaCl_2.2H_2O$, 0.15; $MgSO_4.7H_2O$, 0.2; yeast extract, 0,1; trace element solution – 1 ml. Na-lactate, Na-acetate, Na-propionate, and glycerol (3:1:1:1) – 6 g/l were used as source of carbon and energy for sulphate-reducing bacteria. Uranium (in the form of uranyl acetate), arsenic ($Na_3AsO_4.7H_2O$) and manganese ($MnSO_4.H_2O$) were added separately to the bottles reach to final concentration in the range of 5 to 50 mg/l for the different experiments. Iron ($FeSO_4.7H_2O$) was added in final concentration 50 – 3000 mg/l. pH of inoculated solutions was adjusted to 6,5. The oxygen diffusion was eliminated by 1 cm layer sterile liquid paraffin. The bottles were incubated at 35 $^{o}$C.

### The effect of pH on dissimilatory sulphate reduction

Sulphate-reducing bacteria were grown in a medium with Na-lactate – 6 g/l as a source of carbon and energy. The nutrient solution had the previously mentioned composition. The initial pH of the inoculated solutions was adjusted to levels varying from 2.0 to 7.0 by adding HCl. The bottles were incubated at 30 $^{o}$C.

### The effect of high concentration nitrate on dissimilatory sulphate reduction

Three different kind of medium were used for growth of sulphate-reducing bacteria, depending of source of organic carbon: I - Na-lactate – 10.0 g/l: II – Na – acetate - 10 g/l; III – solid organic matter (mix of cow manure, spent mushroom compost and sawdust) – 100 g/l. The nutrient solution used in these tests contained (in g/l) $Na_2SO_4$ – 3.0; $KH_2PO_4$ – 0.2; $KNO_3$ – 4.9; $(NH_4)_2SO_4$ – 0.5; KCl – 0.5; $MgSO_4.7H_2O$ – 0.1; $FeSO_4.7H_2O$ – 0.1. The concentration of sulphate and nitrate were accordingly 2,5 and 3,0 g/l. pH of solutions was adjusted to 7,0. The bottles were inoculated with mixed enrichment culture of sulphate-reducing bacteria and other metabolically interdependent microorganisms. The bacteria were incubated at 30 $^{o}$C.

### Analytical techniques

The dissolved metal concentrations were determined by ICP spectrophotometry. Uranium concentration was measured photometrically using the arsenazo III reagent. Sulphate and nitrate concentrations were determined photometrically. The content of soluble organic compounds was determined by measuring its oxidativity (by $KMnO_4$).

The identification and enumeration of the microorganisms inhabiting the bottles were carried out by standart microbiological methods.

## RESULTS AND DISCUTION

The data about maximum and average rate of the bacterial sulphate-reduction and inhibition of the process (in %) by used concentration U, As and Mn are represented at table 1. Change of sulphate concentration in presence of As, U and Mn, accordingly 5,15, 25 and 50 mg/l and control (without toxic elements) are represented at figure 1. The maximum rate of sulphate reduction was calculated during logarithmic phase (up to 10 day) of bacterial growth for all tests, whereas the average rate – for overall cultivation (30 days).

The concentrations of U, As and Mn, which were typically for real drainage waters have been no essential reducing influence on the rate of the dissimilatory sulphate reduction.

The concentrations of uranium, below 10 mg/l and manganese – below 15 mg/l have brought to decrease of the rate of sulphate reduction in range 7 to 25% for different experiments. The abovementioned toxic elements have been proved much more negative effect (44 – 52%) in higher concentration – 50 mg/l.

Table 1. The effect of U, As and Mn in different concentration on dissimilatory sulphate reduction

| Element | Contents, mg/l | Maximum rate, mg $SO_4^{2-}$/l.d | Average rate, mg $SO_4^{2-}$/l.d | Iinhibition, % |
|---|---|---|---|---|
| Control | 0 | 98,0 | 74.4 | |
| U | 5 | 85,0 | 69.5 | 7– 14 |
| | 10 | 75.3 | 62.2 | 17 – 24 |
| | 15 | 66.4 | 57.9 | 22 – 32 |
| | 25 | 57,6 | 46,5 | 38 – 42 |
| | 50 | 47,3 | 40,6 | 46 – 52 |
| As | 5 | 81.2 | 64.2 | 14 – 17 |
| | 10 | 73.7 | 57.8 | 25 – 32 |
| | 15 | 69.8 | 48.4 | 29 – 35 |
| | 25 | 60,5 | 41,6 | 41 – 45 |
| | 50 | 51,0 | 31,7 | 48 – 57 |
| Mn | 5 | 89.2 | 69.2 | 7 – 9 |
| | 10 | 82.0 | 66.8 | 10 – 16 |
| | 15 | 74.0 | 60.3 | 19 – 25 |
| | 25 | 63,7 | 54,6 | 26 – 35 |
| | 50 | 52,3 | 41,7 | 44 - 47 |

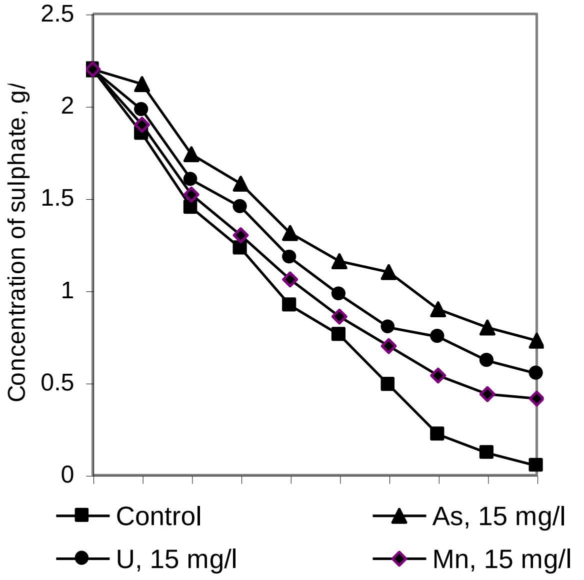

*Figure 1. The effect on U, As and Mn, 15 mg/l on dissimilative sulphate reduction*

It was found that arsenate ions have most toxic effect on sulphate reducing bacteria. Even low concentration of $As^{5+}$ - 5 – 10 mg/l has bought to abatement of the process rate with 14 – 32%, while in presence of 50 mg As/l the rate of sulphate reduction has fallen more than twice.

The cat ions of arsenic and manganese also had negative effect on growth of sulphate reducing bacteria. It was counted maximum number of microorganisms – $10^4$ – $10^6$ cells/ml at stationary phase, while their number in control was $10^7$ cell/ml. It wasn't found influence of studied concentrations uranium on number of sulphate reducing bacteria independent of measured lower rate of sulphate reduction.

The concentrations of dissolved toxic elements were decrease by carry out of dissimilatory sulphate reduction. Sulphate reducing bacteria enzimatically reduced highly soluble hexavalent uranium, wherefore it was found efficiently precipitation (98 – 99%) of radioactive element as far back as 20 day. The cat ionic analyses showed gradual decrease of concentrations of arsenic and manganese during the experiments. Lower concentration of $Mn^{2+}$ measured at the end of test probably to be due to processes such as biosorption and bioaccumulation by present micro flora, as well as by formation of $MnCO_3$. Reducing conditions in medium didn't allow oxidation of $Mn^{2+}$ and formation of insoluble $MnO_2$. Major part of arsenic was removed by means of both precipitation of $As_2S_3$ and $As_2S_5$, and sorption by microbial biomass. It was found 60 – 80% removal of manganese at the end of different experiments.

The concentrations of ferrous ions in range 0,5 to 2 g/l were raised the rate of sulphate reduction in difference of U, Mn and As, because $Fe^{2+}$ consumed microbial generated $H_2S$, which is toxic for sulphate reducing bacteria in high concentration. It was measured highest rate of the process - 118 mg $SO_4^{2-}$/l.d in the presence of 1,5 g Fe/l at first to 21 day of cultivation. Subsequently it was found the lack of change of sulphate concentration by reason of exhausted source of carbon and energy for sulphate reducing bacteria (figure 2). Concentration of ferrous ions about 3 g/l lead to materially reduce of the microbial sulphate reduction (42%) because of partial oxidized of $Fe^{2+}$, succeeded of hydrolyze and precipitation of $Fe(OH)_3$, as well as increased of Eh of medium to unfavourable value for carry out of the process.

It was found efficiently precipitation of iron in form of FeS (96,5 – 99%), when initial concentration of ferrous ions was 0,5 – 1,5 g/l.

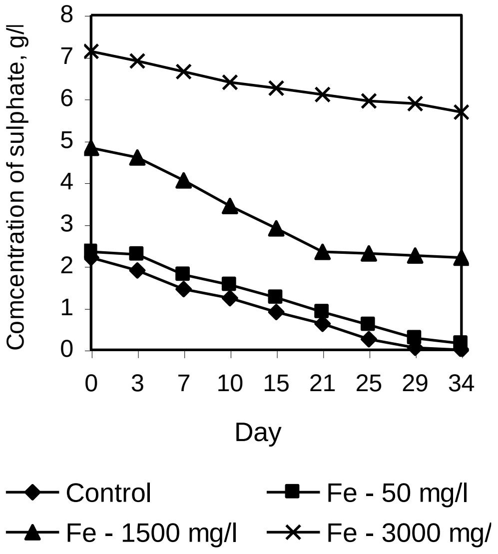

*Figure 2. The effect of $Fe^{2+}$ on dissimilative microbial sulphate reduction.*

Contents of 3 g Fe/l decreased both the rate of microbial sulphate reduction and growth of sulphate reducing bacteria – their number in stationary phase was only $10^4$ cells/ml. Amount of microbial generated $H_2S$ was not enough to react with all quantity ferrous ions – only 39% of iron was precipitated at the end of experiment.

**The effect of pH on dissimilative microbial sulphate-reduction**

It was observed neutralization of medium independently of initial values of pH in all experiments.

The change of acidity at time was due to both of microbial sulphate-reduction, related with producing of bicarbonate ions (when pH is neutral or slight acid) and amonification of rich of proteins yeast extract, involved in medium of anaerobic microflora.

The highest rate of dissimilative sulphate-reduction - 94 mg $SO_4^{2-}$/l.d was measured to 17 day from beginning of experiment in case of initial pH 7(Figure 4). The rate of sulphate-reduction on slightly acid level was in range 50 to 80 mg $SO_4^{2-}$/l.d.

Sulphate reducing bacteria had activity after increasing pH of medium above 4,9 – 5,1 for different experiments. It was found very low rate of sulfate-reduction when initial pH of solutions were strongly acid. The process was began after 24 day, when the conditions for growth of sulphate reducing bacteria was unfavorable because a part of soluble organic matter was used of remaining anaerobic heterotrophic microflora.

It was enumerated highest number of sulphate reducing bacteria at stationary phase – $10^7$ – $10^8$ cells/ml in cases of initial pH values from 5.0 to 7.0.Their number was in range $10^2$ – $10^3$ cells/ml for the rest of experiments.

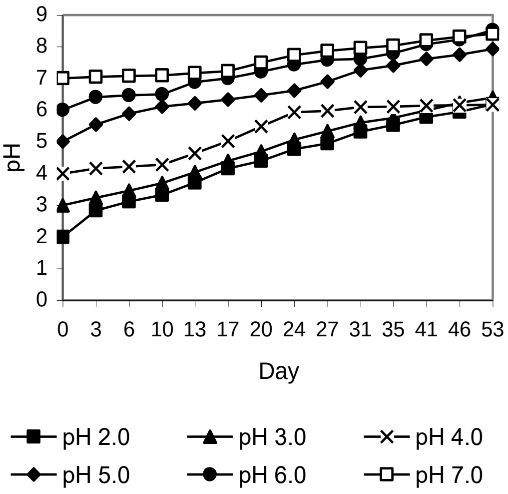

*Figure 3. Dynamic of pH of medium during microbial sulphate reduction*

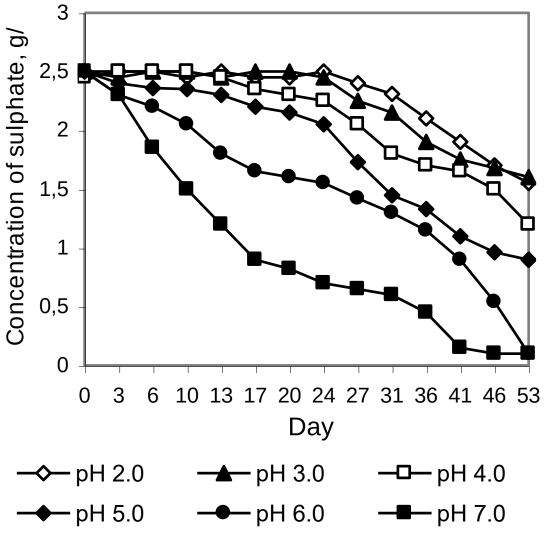

*Figure 4. The effect of pH on dissimilative microbial sulphate reduction*

**The effect of high concentration of nitrate on dissimilatory sulphate reduction**

It was found dominance of reduction of nitrate at concomitant carried out of sulphate-reduction and denitrification. Microbial sulphate-reduction was began after decreased nitrate concentration below 0,5 g/l for all tests (Figure 5).The type of used sources of carbon and energy influenced on the rate of both processes. It was measured highest rate of nitrate reduction - 400 mg $NO_3^-$/l.d at the beginning of experiment with used of acetate

Maximum rate of sulphate-reduction - 80 mg $SO_4^{2-}$/l.d was estimated with used of waste organic matter (mix of cow manure, spent mushroom compost and sawdust) as source of carbon and energy. The sulphate concentration was increased to 4,2 g/l at beginning of experiment because dissolution of gypsum, which have been added in mushroom compost preparation.

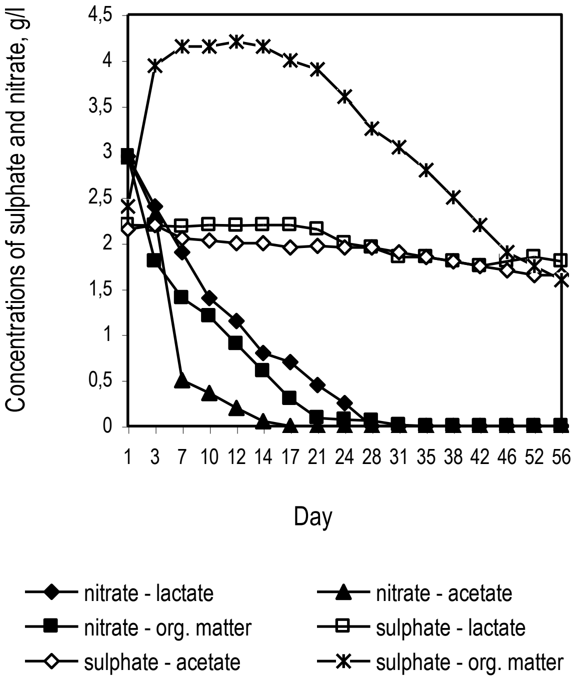

*Figure 5. Dynamic of the concentrations of sulphate and nitrate at concomitant carried out of sulphate and nitrate reduction.*

Insoluble biopolymers (cellulose, hemicellulose and etc.) in anaerobic conditions were slowly digested by complex of metaboliticaly related microorganisms. As a result of this the concentrations of soluble organic acids and alcohols (donors of electrons for sulphate reducing bacteria) were high. The microbial sulphate reduction was limited for remaining experiments as result of exhausted of assimilated organic during the process of genitrification, as well as increased of pH above 8,5 – 9,0.The microflora date revealed dynamics in population density involved in complex microbial cenoses. At the beginning the denitrified bacteria were dominant (their number reached $10^6$ – $10^7$ cells/ml at 20 day). The sulphate reducing bacteria number started to increase after 30 day and reach $10^5$ – $10^7$ cells/ml. It was found a large number of cellulolytic, fermenting bacteria and other anaerobic microorganisms in the liquid phase of waste organic matter.

## CONCLUSIONS

The sulphate reducing bacteria showed high resistance to ionic forms of U, Mn and As. The process carried out with rate, which enable generation of high concentrations $H_2S$ even at concentration of toxic elements 50 mg/l, which are much then real of drainage waters of mining areas. The strong reducing agent has precipitated most of heavy metals as insoluble sulfides. The concentration of hexavalent uranium in range 5 to 50 mg/l has efficiently removed (98 – 99%) as insoluble uraninite by microbial sulphate reduction. The presence of ferrous ions in range 40 – 2 000 mg/l stimulated both the process and its efficiency.

The bicarbonate ions generated by activity of sulphate-reducing bacteria lead to increase of pH in surround environment. After forming of these microzones with neutral pH is possible to outgoing of the process even in strong acid drainage waters and precipitation of some pollutants in the form as hydroxides.

The process of microbial sulphate reduction has been inhibited completely in presence of nitrates in concentration above 0.5 g/ l. This limitation has been important at the biological treatment of mixed acid drainage waters with sewage/ industrial waters, which contain high concentration of nitrates.

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