

Influence of electron donors and copper concentration on geochemical and mineralogical processes under conditions of biological sulphate reduction

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ABSTRACT:

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Sulphidogenous microorganism communities were isolated from soil polluted by crude oil. The study was focused on determining the influence of 1) copper (II) concentration on the activity of selected microorganism communities and 2) the applied electron donor on the course and evolution of mineral-forming processes under conditions favouring growth of sulphate-reducing bacteria (SRB). The influence of copper concentration on the activity of selected microorganism communities and the type of mineral phases formed was determined during experiments in which copper (II) chloride at concentrations of 0.1, 0.2, 0.5 and 0.7 g/L was added to SRB cultures. The experiments were performed in two variants: with ethanol (4 g/L) or lactate (4 g/L) as the sole carbon source. In order to determine the taxonomic composition of the selected microorganism communities, the 16S rRNA method was used. Results of this analysis confirmed the presence of *Desulfovibrio*, *Desulfohalobium*, *Desulfotalea*, *Thermotoga*, *Solibacter*, *Gramella*, *Anaeromyxobacter* and *Myxococcus* sp. in the stationary cultures. The post-culture sediments contained covellite (CuS) and digenite (Cu₉S₅). Based on the results, it can be stated that the type of carbon source applied during incubation plays a crucial role in determining the mineral composition of the post-culture sediments. Thus, regardless of the amount of copper ion introduced to a culture with lactate as the sole carbon source, no copper sulphide was observed in the post-culture sediments. Cultures with ethanol as the sole carbon source, on the other hand, yielded covellite or digenite in all post-culture sediments.

Key words: Anaerobic conditions; Biogenic minerals; Copper; Geochemical processes; Sulphate-reducing bacteria.

INTRODUCTION

The role of microorganisms that significantly influence the nature and course of mineral- and rock-forming processes in the hypergenic environment is stimulating interest among earth scientists. There is a mutual relationship between the biotic and abiotic elements in this environment. Various mineral phases that are formed in biological processes may significantly influence the activity and metabolism of microorganisms as well as the physical and chemical

properties of the environment in which they occur. Many reports devoted to the crystallization of mineral phases, the development of rock- and deposit-forming processes and sedimentation under hypergenic conditions have supplied information on the role of microorganisms in the formation of carbonates, sulphides or elemental sulphur in anaerobic conditions; however, the data are very scanty, and the reports usually only briefly refer to the issues (Popa *et al.* 2004; Borkowski and Wolicka 2007a, b; Wolicka and Borkowski 2008).

The formation of mineral phases containing various heavy metals, including sulphides, should be discussed with regard to biogeochemical processes. According to the commonly accepted model, the most important processes of sulphide formation in hypergenic settings, leading to the formation of such important metal deposits as those of iron, copper, zinc or lead, for example, are abiotic chemical reactions (Gramp *et al.* 2006, 2010; Sarradin *et al.* 2007). However, these are not the only processes by which metal sulphides of low solubility may be formed. Sulphides also develop through biochemical reactions as products of microbiological activity in anaerobic conditions. Some metal sulphides in sedimentary rocks are evidently of biogenic origin (Gould *et al.* 1997; Gibson 1990). The main process leading to the formation of biogenic sulphides is biological sulphate reduction. Reduction of sulphate ions results in the formation of sulphide ions, which form sulphides with particular metal cations (Rickard and Luther 2007).

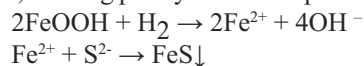
Sulphate-reducing bacteria (SRB) that are heterotrophs and absolute anaerobes play an important role in the process of sulphate reduction. They utilize sulphates and other oxidized sulphur compounds as the final electron acceptors in respiration processes (Widdel and Rabus 2001). The preferred carbon sources for SRB are usually low-molecular-weight organic acids, e.g. acetic acid and propanoic acid, alcohols, e.g. methanol (Fauque *et al.* 1988, 1991), and aliphatic and aromatic hydrocarbons (Kleikemper *et al.* 2002; Wolicka 2009a, b). Apart from easily accessible carbon sources and the presence of oxidized sulphur compounds, many physical and chemical factors influence the growth and life of sulphidogenic microorganism communities. These include the concentration of dissolved oxygen in the environment, temperature, pH, Eh and the presence of an accompanying microflora. The variable physiology of SRB allows for their presence in both natural and anthropogenically affected environments. SRB also occur in extreme environments, such as crude oil, deposit waters and soils polluted by heavy metals (Reuter *et al.* 1994). The presence of SRB in these environments is very important not only for their possible application in the neutralization or passivation of hazardous compounds and revitalization of the soil environment, but most importantly for their significant role in the mineral-forming processes taking place in the hypergenic environment, e.g. during the formation of deposits of various sulphides.

The presence of heavy metals in the environment often hampers the growth and development of autochthonous microorganisms. However, the toxicity of

a given metal depends not only on its presence in the environment, but also on its chemical form and concentration. Available reports do not supply data that describe either the toxicity of heavy metals and their influence on SRB activity or the qualitative composition of minerals formed during metabolic processes of SRB.

It was long considered that the significant toxicity of many heavy metals does not allow microbiological precipitation of sulphides by SRB. As early as 1961, Baas-Becking and Moore conducted an experiment in which salts of selected metals were added to a medium with a composition close to that of sea water. The results unequivocally pointed to the role of SRB in the formation of secondary mineral phases in the analyzed system. For example, the product of biotransformation of FeSO₄ was pyrite (FeS₂), that of malachite (Cu₂(CO₃)(OH)₂) was covellite (CuS), and that of smithsonite (ZnCO₃) was sphalerite (ZnS).

Apart from SRB, a significant role in the precipitation of sulphides is played by iron (III)- or manganese-reducing bacteria (Lovley 1991, 1995). By the activity of these two microorganism communities, the environment is supplied with ions of metals at a lower oxidation level, which then may react with sulphide ions, forming poorly soluble sulphides of these metals:



The aim of the present study was to determine the influence of copper (II) concentration on the activity of selected microorganism groups and the applied electron donor on the direction and course of mineral-forming processes under conditions favoring SRB development.

MATERIAL AND METHODS

Selection and isolation of sulphidogenous microorganism cultures

The microorganisms were isolated from soil polluted with crude oil and oil-derived products. First, an Easycult S test was made to check for the presence of sulphidogenous microorganism communities; next, the microorganisms were selected and grown using the *microcosms* method. Soil samples (10 g) from the study environments were inserted in 100-ml flasks and covered with a particular medium. Two types of media were applied: a modified Postgate C medium (without yeast extract and sodium citrate) with lactate and ethanol as the sole carbon source and a minimal medium. The flasks were tightly closed and incubated in darkness for 6

weeks at a room temperature of about 25°C in order to select anaerobic, sulphidogenous microorganism communities capable of simultaneous biodegradation of the applied carbon sources and sulphate reduction.

Anaerobic stationary cultures were carried out in 0.5-L glass bottles filled to $\frac{3}{4}$ volume and tightly sealed with rubber corks that were pierced with needles connected permanently to syringes that were used to introduce the inoculum and to collect samples. The inoculum to medium ratio was 1:10.

The anaerobic conditions in the cultures were controlled by addition of resazurin as the oxygen-level indicator. A violet color indicated that the culture contained oxygen; its absence pointed to anaerobic conditions.

Media

A modified liquid Postgate C medium (Postgate 1984), with composition KH_2PO_4 (0.5 g/L), NH_4Cl (1.0 g/L), CaCl_2 (0.06 g/L), MgSO_4 (0.06 g/L), FeSO_4 (0.1 g/L), Na_2SO_4 (4.5 g/L) without yeast extract and citrate, and a minimal medium (NH_4Cl – 1 g/L, Na_2SO_4 – 4.5 g/L) were applied in the experiments. Lactate (4 g/L) or/and ethanol (4 g/L) were added to both media as the sole carbon source. Resazurin (0.001 g/L) was added to all cultures to control the oxidation of the medium.

Chemical determinations

Sulphate determinations were made using the turbidimetric method after reaction with barium chloride in a Thermo spectrophotometer at $\lambda = 400$ nm wavelength.

Protein determinations in the cultures were made using the Lowry method after a biuret test enhanced by the Folin-Ciocalteu reagent in a Thermo spectrophotometer at $\lambda = 670$ nm wavelength.

Determination of COD values (chemical oxygen demand) was made using the dichromate method, oxidizing organic compounds or some inorganic compounds at a temperature of 168°C with the application of a mixture composed of $\text{K}_2\text{Cr}_2\text{O}_7$, H_2SO_4 , and H_3PO_4 in the presence of Ag_2SO_4 as catalyst and titration of the excess dichromate by the Mohr salt against ferroin. COD use by SRB was calculated according to $\text{COD}/\text{SO}_4 = 0.67$ [g/g] (Hao 1996).

Influence of copper ions on SRB activity. In order to determine the influence of copper concentration on the

activity of selected microorganism communities and the type of mineral phases formed, an experiment was conducted in which copper (II) chloride in different concentrations was added to the SRB cultures. The experiments were carried out with either ethanol or lactate as the sole carbon source (4 g/L). The medium did not contain a yeast extract or sodium citrate. Copper (II) chloride was added in concentrations of 0.1, 0.2, 0.5 and 0.7 g/L to the modified Postgate C medium. The inoculum applied was a selected SRB community isolated from soil polluted with crude oil and containing an increased copper (II) concentration. The experiment was repeated twice. The control batch comprised stationary SRB cultures without copper.

Copper determinations in stationary cultures. The determinations were made using the Thermo spectrophotometer with the application of available kits for determining copper concentrations (0.02 – 6.00 mg/L, Spectroquant, Merck).

Mineralogical analysis of post-culture sediments

After incubation the cultures were centrifuged at 14000 rpm, and the post-culture deposit was dried at 30°C without oxygen supply. The samples were next ground in an agate mortar, and their mineral composition was determined using X-ray powder diffraction in an X Pert PRO MPD diffractometer. Depending on the content of the deposit, the diffraction measurement was made in a capillary (DSH) or in a classical Bragg–Brentano system.

Molecular analysis of selected sulphidogenous microorganism communities

The taxonomic composition of the selected SRB communities was determined using molecular analysis. Isolation of chromosome DNA and analysis of gene 16S rRNA fragments were carried out according to commonly applied procedures (Collins *et al.* 1991). Bacterial DNA was isolated from a fluid culture of microorganisms by application of a commercial kit for chromosomal DNA isolation. The purity and concentration of the obtained DNA preparation were determined spectrophotometrically at 260 nm. Primers specific for bacterial 16S rRNA (27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3') were used to amplify a 1540-bp segment from the 16S rRNA gene. The PCR reaction was made using the GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Invitrogen). Amplification products were purified using the Wizard Purification System

(Promega) and analyzed by electrophoresis. After amplification, the material was sequenced using the ABI 3730 Genetic Analyzer with application of the Perkin Elmer sequencing kit. The obtained nucleotide sequences were compared to gene 16S rRNA sequences available in the National Centre for Biotechnology Information (NCBI) database using the Blast 2.0 program.

RESULTS AND DISCUSSION

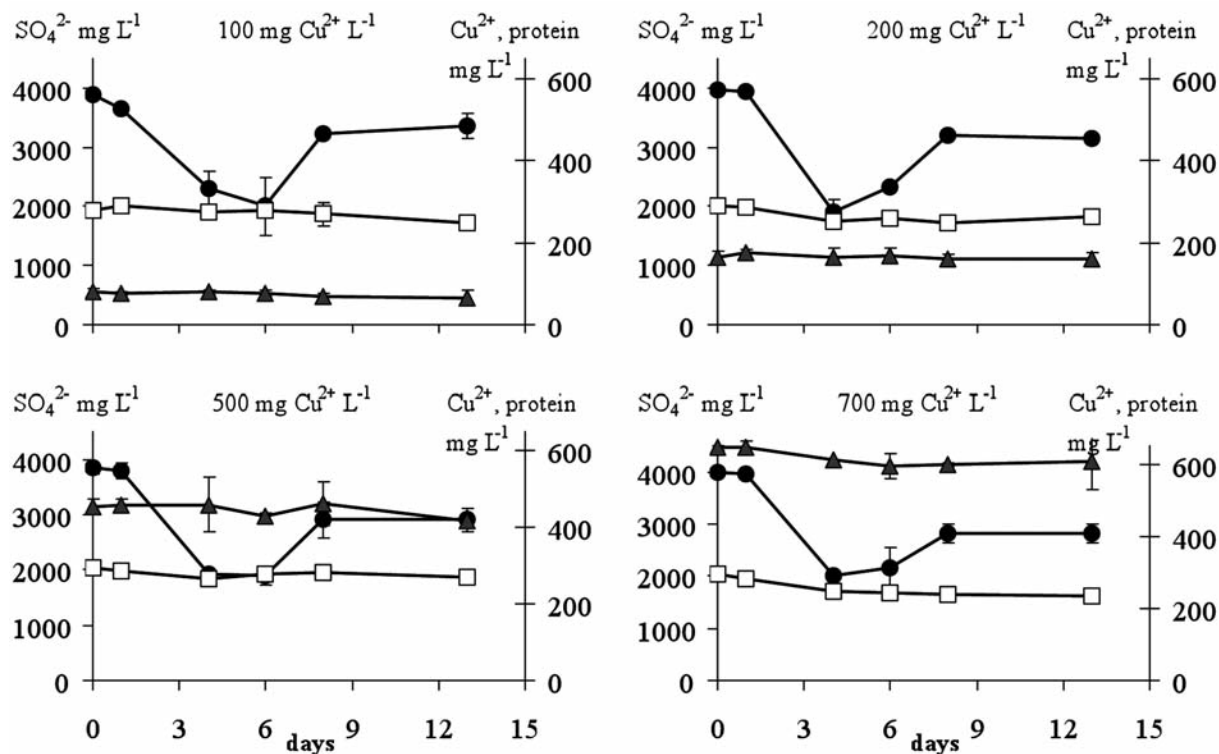
The source of microorganisms was soil polluted with crude oil, in which increased concentrations of copper were noted. In order to obtain high activity, cultures of sulphidogenous microorganisms underwent multiple passaging on a Postgate medium with lactate and ethanol as the sole carbon sources.

The influence of copper concentration on the activity of selected SRB communities from soils polluted with crude oil and oil-derived products was tested in stationary cultures containing various concentrations of copper ions, from 0.1 to 0.7 g/L, and control cultures for the experimental system that did not contain copper. The carbon sources applied were low-molecular-weight compounds such as lactate and ethanol, which are easily accessible to SRB.

At first, a considerable fall in sulphate concentration was observed in all cultures; next, after a week-long incubation, an increase was noted. A possible cause of this increase is abiotic hydrogen sulphide oxidation (Text-figs 1, 2). A similar trend was noted in the control batch (Text-fig. 3).

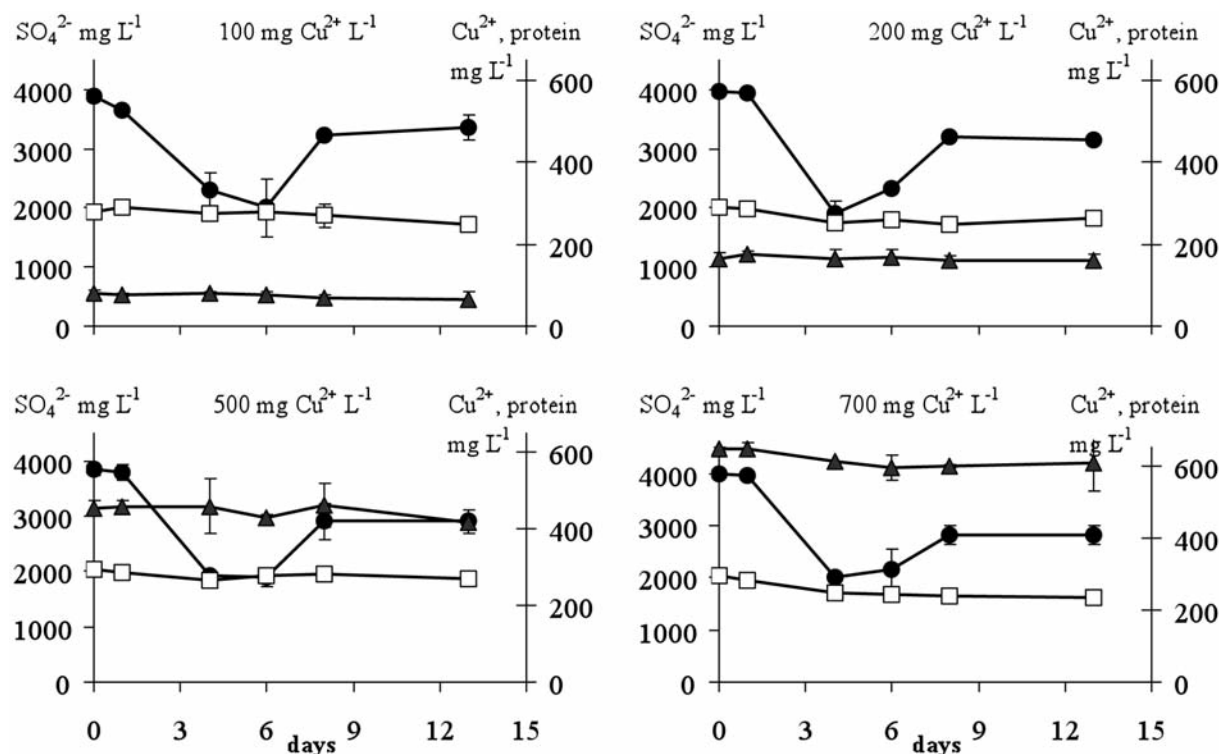
The concentration of protein in the control cultures indicated a distinct initial increase in the abundance of the microorganisms, whereas in cultures with copper, this concentration remained at approximately the same level. This probably points to the inhibiting effect of copper ions on some microorganisms within the analyzed microorganism community, whereas the presence of copper, even at a concentration of 0.7 g/L, did not inhibit sulphate reduction by SRB.

For most organisms, copper is a potentially hazardous ion, and copper toxicity is lowered by increasing pH and by the presence of organic compounds. According to Sani *et al.* (2001), IC_{50} (concentration causing growth inhibition for 50% of the cells) for *Desulfovibrio desulfuricans* G20 is 1 mg Cu^{2+}/L . Sani *et al.* (2001) also cite Utgikar *et al.* (2001), who report that EC_{50} (concentration causing 50% reduction of the sulphidogenous activity of SRB) reaches 10.5 mg Cu^{2+}/L , and Panchanadikar and Kar (1993), who suggest that a copper concentration of 100 ppm inhibited biological precipitation.



Text-fig.1. The concentration changes of sulphate, protein and cuprum in sulphidogenous microbial cultures with an ethanol as a sole organic carbon source, at the different initial concentration of Cu^{2+} (100, 200, 500, 700 mg L^{-1}). Symbols: ● - sulphate, □ - protein, ▲ - copper. Standard deviation has been marked

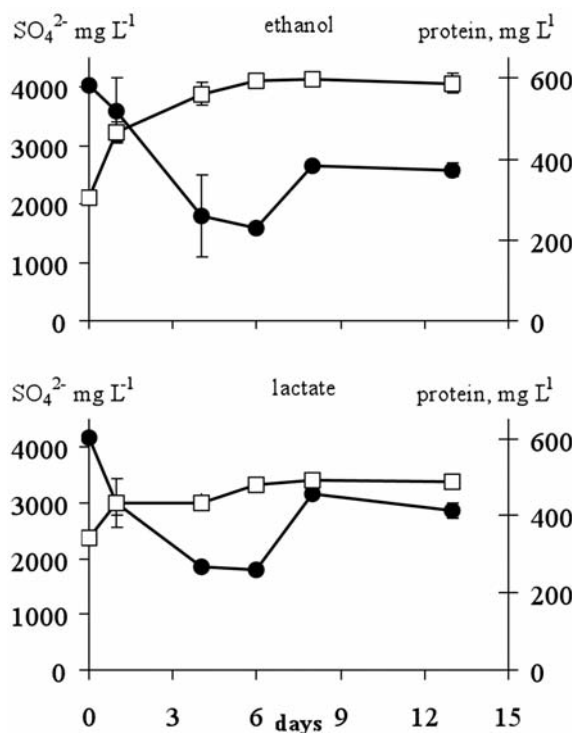
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Text-fig. 2. The concentration changes of sulphate, protein and cuprum in sulphidogenic microbial cultures with a lactate as a sole organic carbon source, at the different initial concentration of Cu^{2+} (100, 200, 500, 700 mg L⁻¹). Symbols: ● - sulphate, □ - protein, ▲ - copper. Standard deviation has been marked

These data do not contradict the results obtained in the present study. It seems, however, that they may be underestimates. Most probably, the presence of copper ions in an environment favouring SRB growth does not have a high inhibiting effect, as suggested by literature data. In the present experiments, copper was added at concentrations of 1.57–11.0 mmol/L to cultures of selected communities with lactate and ethanol as the sole carbon sources. In experiments described in previous reports, the concentration of copper was much lower than this. For example, Gramp *et al.* (2006) conducted an experiment in which 8.95 mmol/L Cu (II) and sodium sulphate (VI) were added to a medium containing sodium citrate, yeast extract and sodium lactate as sources of carbon. In this case, only covelline was noted in the post-culture sediments as the product of copper biotransformation.

The results of genetic analysis of selected sulphidogenic microorganism communities confirmed the presence of the following bacterial strains: *Desulfovibrio vulgaris* DP4, *Desulfovibrio salexigens* 2638, *Desulfohalobium retbaense* DSM 5692, *Desulfovibrio vulgaris* str Miyazaki F, *Thermotoga lettingae* TMO and *Solibacter usitatus ellin* 6076 in stationary cultures on medium with ethanol as the sole carbon source, and *Desulfotalea psychrophila* LSv54, *Gramella forsettii* KT 080-3, *Desulfovibrio desulfuricans* str ATCC27774, *Anaeromyxobac-*



Text-fig. 3. The concentration changes of sulphate and protein in the control (without addition of Cu^{2+}) sulphidogenic microbial cultures with an ethanol or lactate as a sole organic carbon source. Symbols: ● - sulphate, □ - protein. Standard deviation has been marked

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copper (II) concentration	Source of carbon	
	lactate	ethanol
0.1 g/L	anhydrite CaSO_4 , euchlorine $\text{KNaCu}_3(\text{SO}_4)_3\text{O}$	sulfur S, mackinawite FeS , wooldridgeite $\text{Na}_2\text{CaCu}_2(\text{P}_2\text{O}_7)_2 \times 10\text{H}_2\text{O}$, digenite $\text{Cu}_{1.8}\text{S}$
0.2 g/L	smithite $\text{Fe}_{13}\text{S}_{16}$, euchlorine $\text{KNaCu}_3(\text{SO}_4)_3\text{O}$, greigite Fe_3S_4 , plypite $\text{K}_4\text{Cu}_4(\text{Na,Cu}^+)(\text{SO}_4)_4\text{ClO}_2$	sulphur S, covellite CuS
0.5 g/L	smithite $\text{Fe}_{13}\text{S}_{16}$, euchlorine $\text{KNaCu}_3(\text{SO}_4)_3\text{O}$ greigite Fe_3S_4	sulphur S, covellite CuS
0.7 g/L	sulphur S	sulphur S, greigite FeS , digenite $\text{Cu}_{1.8}\text{S}$, phosphofibrite $\text{KCuFe}_{15}(\text{PO}_4)_{12}(\text{OH})_{12} \times 12\text{H}_2\text{O}$

Table 1. Results of analysis of post-culture sediments in SRB cultures with copper (II) chloride on modified Postgate C medium

ter sp. and *K. Myxococcus xanthus* DK 1622 on medium with lactate. Precipitation of copper by *Desulphovibrio* sp. was reported by Panchanadikar and Kar (1993).

The sulphidogenous microorganism communities showed activity even at copper concentrations of 0.7 g/L, which was confirmed by diffractometric analysis of the post-culture sediments, in which copper sulphide was present, and mainly by analysis of the sulphide concentrations in the cultures, which showed a significant reduction of sulphide ion concentration both in the control and in the experimental batches.

SRB activity in the presence of copper ions is reflected and confirmed by the presence of sulphur, copper sulphides such as covellite and digenite, and the iron sulphides mackinawite, smithite and greigite in the post-culture sediments (Text-figs 4, 5).

According to Gramp *et al.* (2006), covellite of biogenic origin and sulphides of other metals do not have distinct crystalline structures like that of covellite of chemical origin. Moreover, poorly crystalline biogenic covellite is more susceptible to biological oxidation compared to the more resistant abiotic covellite. Two minerals, covellite (CuS) or chalcocite (Cu_2S), are formed from a solution that contains sulphide and copper ions. According to Gramp *et al.* (2006), covellite is formed when there is a larger amount of sulphide ions than copper ions in the environment; a higher concentration of copper than sulphide ions results in the formation of chalcocite.

The post-culture sediments of the SRB cultures pointed to the presence of covellite (CuS) and digenite (Cu_9S_5). Based on our results, it can be assumed that the mineral composition of the post-culture sediments mainly depends on the type of carbon source applied during incubation (Table 1). Regardless of the amount of copper ions introduced to the cultures with lactate as the sole carbon source, the post-culture sediments did not yield any copper sulphide. In cultures with ethanol as the sole carbon source, all post-culture sediments showed the presence of covellite (CuS) or digenite (Cu_9S_5). Thus it can be assumed that the chemical composition of the post-culture sediments depends on the type of electron donor applied (Text-figs 4, 5).

The experimental results show that biogenic synthesis of metal sulphides in soils polluted by oil-derived products is possible even at high concentrations of heavy metals. Taking into account the XRD results, it can be observed that the types of mineral phase formed depend on the type of applied carbon source (Table 1). A very important result obtained during the present experiments is the existence of copper sulphide in the post-culture sediments in all cultures with ethanol as the sole carbon source, regardless of the copper (II) concentration in the culture.

The main reason why different mineral phases are formed is the presence of the so-called microniches, i.e. syntrophic systems that very often allow the growth of a specific group of microorganisms at the molecu-

lar level. Their presence in the soil environment is relatively common; however, identical microorganism communities do not develop in every system.

During the formation of metal sulphides, the selection and growth of microorganisms depend mainly on the concentration of the cation within a given sulphide. Very often metal cations have a toxic influence on microorganism activity, and in some cases they inhibit biochemical reactions taking place under the influence of a given microorganism group. The formation of mineral phases in the presence of microorganisms need not be directly determined by the general physical and chemical properties of the environment; it may be determined by those characteristic of a small area in the immediate vicinity of a bacterial cell, specific local conditions (biogeochemical microniches) that are the result of local microbiological activity, even when the general environmental conditions would not theoretically allow such mineral formation (Wolicka 2011).

CONCLUSIONS

An important result of the experiments is the existence of copper sulphides in post-culture sediments of all cultures with ethanol as the sole carbon source, regardless of the content of copper (II) in the culture. The activity of sulphate-reducing bacteria was detected in all cultures even with high concentration of copper. These observations are important due to the possibility of formation of sulphide minerals *via* biological activity of microorganisms under anaerobic conditions in the presence of high concentration of copper. The copper ions are regarded as toxic for many microorganisms, but it seems that the presence of sulphate and metals ions in environments under anaerobic conditions can promote growth of the sulphidogenic microbial communities. In all cultures with copper, the concentration of protein did not change or even decreased compared to the controls, possibly due to the toxic effect of copper ions. Consequently high concentration of copper and production of biogenic hydrogen sulphide can strongly affect the growth of other accompanying microorganisms. With regard to environmental protection, SRB have been successfully applied in recent years to the neutralization of acid mine waters that contain cations of heavy metals, including copper. With regard to the existence and course of geochemical and mineral-forming processes, the qualitative composition of the organic matter significantly influences the quality of the mineral phases formed, which may have crucial importance in recognizing the sedimentary conditions of various sediments.

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