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Biological sulfate removal with low-cost carbon sources using cold-acclimated bacteria

Hanna Virpiranta [ID][a,*], Sanna Taskila [ID][a], Tiina Leiviskä [ID][a], Jouko Vepsäläinen[b], Jaakko Rämö[a] and Juha Tanskanen[a]

[a] Chemical Process Engineering, University of Oulu, P.O. Box 4300, 90014 Oulu, Finland
[b] School of Pharmacy, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland
*Corresponding author. E-mail: hanna.virpiranta@oulu.fi

[ID] HV, 0000-0003-4960-5070; ST, 0000-0003-3306-3297; TL, 0000-0002-3130-0060

ABSTRACT

The main goal of this study was to develop a cost-efficient biological method for the removal of sulfate from mining effluents in cold conditions. A consortium of cold-tolerant sulfate-reducing bacteria (SRB) was tested at 6 °C regarding the utilization of economically viable, low-cost carbon sources, i.e., whey, conditioned sewage sludge, and peat, in the removal of sulfate from synthetic mining water. Succinate was used as a reference carbon source. Of all the studied low-cost carbon sources, conditioned sewage sludge proved to be the most efficient. Nuclear magnetic resonance (NMR) spectroscopy revealed that sewage sludge contained propionic acid, which proved to be utilizable by SRB under cold conditions. Peat both adsorbed the sulfate and acted as a nutrient source in the sulfate reduction process. When whey was used as a carbon source, only a slight decrease in sulfate concentration was detected. Succinate was found to work in a truly predictable and efficient way as a carbon source in biological sulfate reduction, even at the lowest concentration tested. The use of conditioned sewage sludge increased the bacterial diversity in liquid cultivations significantly. However, the number of SRB was highest in the succinate cultivations.

Key words: cold-tolerant bacteria, mining, sulfate-reducing bacteria, water pollution

HIGHLIGHTS

- Cold-tolerant sulfate-reducing bacteria (SRB) were successfully cultivated using low-cost carbon sources.
- Kemicond-treated sewage sludge was the most efficient low-cost carbon source.
- Simultaneous sulfate adsorption and reduction were observed with natural peat.
- Organic compounds utilized and produced by the bacteria were characterized by nuclear magnetic resonance (NMR).
- Synthetic mining water was treated to model real water pollution control.

GRAPHICAL ABSTRACT
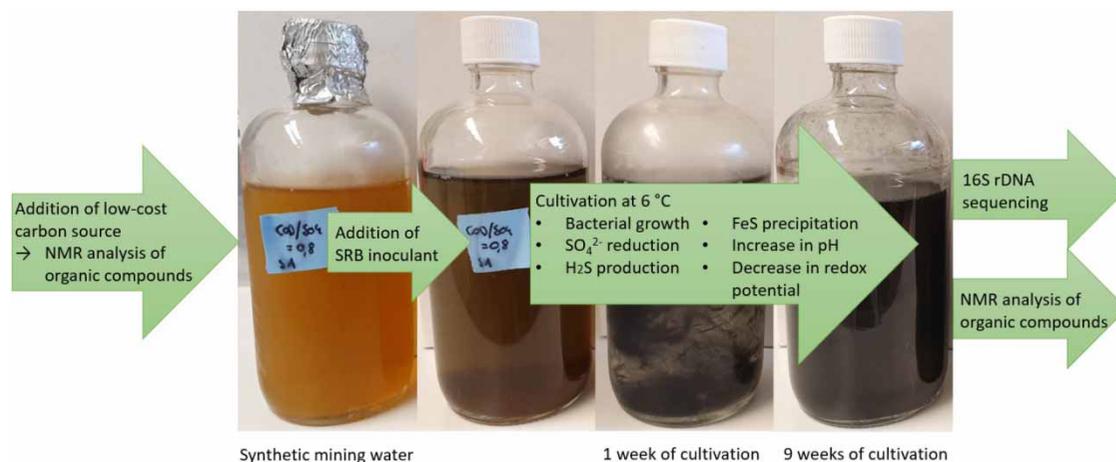
INTRODUCTION

Mining is one of the main causes of the contamination of natural water bodies with sulfate and toxic metals. Growth in rainfall and surface runoffs can even increase the amount of contaminated waters. Further treatment of mining waters has been implemented due to tightening environmental permit conditions. The leakage of untreated mining waters into the environment has also taken place repeatedly around the world. In Finland, the limits for concentrations of sulfate and metals in mining effluent discharge are specific to each mine and the limits for sulfate may vary considerably (Nurmesniemi 2018). The leakage of sulfate-containing effluents into nature can cause enormous environmental problems. Sulfate ions cause the salinization and stratification of fresh waters, which in turn leads to hypolimnetic oxygen depletion (Kauppi et al. 2013). Naturally, the consequences of anoxia are a decrease in biodiversity and changes in aquatic ecosystems (Williams 2001).

Biological sulfate reduction is a widely studied treatment method for liquid waste streams that contain sulfate and metals (Runtti et al. 2018). One advantage of so-called biosulfidogenesis is the simultaneous removal of sulfate and transition metals as sulfide precipitates (Santos & Johnson 2017). However, in northern areas of the northern hemisphere, microbial metabolism is often slow due to the cold climate, which complicates biological wastewater treatment. Nevertheless, by exploiting the native microbes of boreal areas and providing a suitable carbon source, sulfate reducers can succeed even at low temperatures (Nielsen et al. 2018; Virpiranta et al. 2019).

One of the main barriers for implementing biological sulfate reduction on a large scale is the need for an organic carbon source, which has a significant effect on the cost-effectiveness of the treatment (Kaksonen & Puhakka 2007). Therefore, many studies have investigated the use of alternative carbon sources. The utilization of sewage sludge by sulfate-reducing bacteria (SRB) has been tested successfully in several studies (Ristow et al. 2004; Peng et al. 2017; Liu et al. 2018). Liu et al. (2018) fermented sludge and, after centrifugation of the fermentation product, used the supernatant as a carbon source for SRB. Peng et al. (2017) used sewage sludge without any pretreatment and Ristow et al. (2004) sieved the sludge through a 6.7 mm sieve before utilization. Almost complete removal of sulfate was achieved by Ristow et al. (2004), and results were also promising in other studies. The use of whey as a carbon source in biological sulfate reduction had been studied earlier by Wolicka & Borkowski (2009) and Jiménez et al. (2010), achieving adequate sulfate removal rates of 55 and 68%, respectively. Furthermore, Nielsen et al. (2019) tested the utilization of powdered peat at 5 °C in small-scale sulfidogenic bioreactors. However, a sulfate removal rate of only 13.8% was observed, probably because the carbon of such a complex material is poorly bioavailable. Since peat is known as an efficient biosorbent for several contaminants (Zhou et al. 2012; Gogoi et al. 2018, 2019; Leiviskä et al. 2018; Zhang et al. 2019), and exploiting peat soils for development of biosorbents has been extensively investigated (Bartczak et al. 2018; Virpiranta et al. 2020), the role of adsorption in sulfate removal also merits study. In addition, there is a lack of knowledge on the utilization of whey and sewage sludge under cold conditions.

In this study, biological sulfate reduction was investigated at a temperature of 6 °C with low-cost carbon sources – whey from cheese manufacturing, natural peat, and conditioned sewage sludge after acidification, oxidation, and flocculation (the Kemicond process (Cornel et al. 2004; Schaum et al. 2006)). To the best of our knowledge, this is the first study describing the use of this kind of conditioned sludge as a carbon source in a biological sulfate reduction process. In addition, the role of adsorption in sulfate removal was investigated with natural peat. All the low-cost carbon sources used are widely available materials in northern Finland. The peat used in this study was milled fuel peat, which is usually cut from the surface layer of a peat bog, and loosened and pulverized using a miller (Kristoferson & Bokalders 1986). Succinate was used as a reference carbon source, since it had already proved suitable for the SRB consortia used in our previous study (Virpiranta et al. 2019). With every carbon source, COD/sulfate ratios of 0.8, 1.4, and 2.0 were tested to determine the best ratio for efficient sulfate reduction. Dissolved substances were characterized from cultivation liquids by nuclear magnetic resonance (NMR) both before inoculation and after microbial growth to determine which compounds the SRB consortia were utilizing and producing. 16S rRNA sequencing was used to study how the microbial community structure was affected by the use of different carbon sources.

MATERIALS AND METHODS

Sulfate-reducing bacterial consortium

The mixed SRB culture, enriched and cold-acclimated in our previous study (Virpiranta et al. 2019) was found to consist of different anaerobic bacteria – mostly Firmicutes, Bacteroidetes, and Proteobacteria. Approximately 19% of the bacteria were

sulfate reducers and all of the SRB were members of δ-Proteobacteria. The most common SRB genus was *Desulfobulbus*, although some *Desulfovibrio* spp. were also present.

The SRB culture was maintained by cultivation at 6 °C in a modified Postgate medium with succinate as a carbon source. The ingredients of the medium (0.5 g K_2HPO_4, 1.0 g NH_4Cl, 1.0 g Na_2SO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, 2.0 g $MgSO_4 \cdot 7H_2O$, 0.25 g yeast extract, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.2 g ascorbic acid L^{-1}) were dissolved in distilled water, with the exception of sodium succinate. Before autoclaving at 121 °C for 20 min, the pH of the medium was adjusted to 7.8 with NaOH and the solution was distributed into 250 mL glass bottles that were sealed with screw caps and septa. After autoclaving and cooling to 6 °C, sodium succinate solution was added with a syringe and needle so that the succinate concentration in the medium was 1.75 g L^{-1}. All the chemicals used in the media were of laboratory grade.

Sulfate reduction using low-cost carbon sources

The testing of low-cost carbon sources (conditioned sewage sludge, peat, and whey from cheese manufacturing) and different COD/sulfate ratios (0.8, 1.4, and 2.0) was conducted by cultivating the bacteria in synthetic mining wastewater (1.0 g Na_2SO_4, 0.1 g $CaSO_4 \cdot 2H_2O$, 2.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2SO_4, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.2 g ascorbic acid L^{-1}) mixed with suitable amounts of the low-cost carbon sources. Iron was added to the water as an SRB growth indicator, since SRB are more tolerant to iron than other transition metals commonly present in mining waters (e.g., Cu, Ni, and Zn). The synthetic mining wastewater contained 1.96 g L^{-1} sulfate and did not mimic any actual mining water. Succinate was used as a reference carbon source and 0.04–0.1 g L^{-1} of K_2HPO_4, 0.19–0.49 g L^{-1} of NH_4Cl, and 0.1–0.25 g L^{-1} of yeast extract were used as sources of nitrogen and phosphate. Three parallel cultivations were conducted with each carbon source and at each COD/sulfate ratio.

Sewage sludge disinfected and dewatered by the Kemicond process was obtained from Oulu Waterworks, Taskila wastewater treatment plant, which is mainly responsible for the treatment of wastewater coming from households and a few large individual operators including Oulu University Hospital, a dairy facility, fish processing plant, and municipal waste management company. Peat was obtained from Stora Enso Veitsiluoto pulp mill, which uses peat for energy production. The peat was dried at 80 °C for 24 h and then sieved. In this study, a peat fraction of <90 μm was used. The whey from cheese manufacturing that we used was the liquid formed in lactose crystallization at the Kuusamon Juusto cheese processing plant.

The initial sulfate concentration of the mining water was approximately 2 g L^{-1}. After adjusting the pH of the synthetic mining water to 7.8 with NaOH solution, the water was distributed into 250 mL septum screw cap bottles and autoclaved. The carbon sources – succinate, sewage sludge, and whey – and 0.5 g L^{-1} of $FeSO_4 \cdot 7H_2O$ as a visual SRB growth indicator were added after autoclaving. Peat had already been added to the mining water before autoclaving to enhance the settling of peat and dissolving of organic substances from the peat. The bottles were inoculated with 5 mL aliquots of the SRB maintenance culture and inoculated at 6 °C for 2 months. No additional carbon sources were added during the incubation period.

To investigate the sulfate adsorption capacity of the peat, synthetic mining water was mixed with different amounts of peat in a horizontal rotary shaker (20 rpm) for 3 days at 6 °C without a bacterial inoculum. At the end of the incubation period, each mixture was centrifuged (6 min, 10,000 RCF) and the residual sulfate concentration was analyzed from the supernatant. The adsorption experiment was conducted both with autoclaved peat as well as with a non-treated peat solution to test the effect of autoclaving on the adsorption capacity of the peat.

Sampling and analysis

The COD values of the low-cost carbon sources were measured with the following Hach Lange COD cuvette tests: LCK 014 (sewage sludge), LCK 514 (peat solution of 3.5 g L^{-1}), and LCK 914 (whey). The total dissolved nitrogen and phosphorus of the sewage sludge, peat solution, and whey were measured with Hach Lange cuvette tests LCK 338 and LCK 350, respectively. The carbon source suspensions were decanted and samples for the cuvette tests were taken from the liquid phase. The elemental composition (carbon, hydrogen, and nitrogen) was determined for the peat fraction using a 2400 Series II CHNS/O analyzer (PerkinElmer). A Precisa prepASH 129 furnace was used to measure the ash content of the peat at 525 and 950 °C. The initial lactic acid and lactose concentrations of the whey were measured with a YSI 2700 Select Biochemistry Analyzer (YSI Life Sciences 2009). The measurement is based on the changes in electrical current that are formed when the examined substance is enzymatically broken down on a membrane. For the lactose measurements, β-galactosidase from *Aspergillus oryzae* was used to hydrolyze the lactose, after which the glucose concentrations were measured.

Samples of the culture solutions were collected periodically through the septa with a syringe and needle, and centrifuged for 6 min at 10,000 RCF and 4 °C. The pH, redox potential, and sulfate concentration were measured from the supernatant.

An IQ150 pH Dual Technology meter equipped with a Mettler Toledo InLab Flex-Micro pH electrode or pHenomenal ORP220 Ag/AgCl (3 M KCl) electrode was used for measuring the pH and redox potential, respectively. For reference to a standard hydrogen electrode, $+215$ mV (AgCl/Ag potential at 10 °C) was added to the recorded redox potentials. Hach Lange Sulfate cuvette test LCK 353 and a UV/Vis Spectrophotometer DR 2800 were used for determining the sulfate concentrations.

The NMR spectra of synthetic mining waters supplemented with the carbon sources were measured before inoculation and after the cultivations using a 600 MHz Bruker NMR spectrometer, equipped with a cryoprobe (Bruker Prodigy TCI 600 S3 H&F-C/N-D-05 Z) and an automatic SampleJet sample changer. Prior to the NMR measurements, 200 μL of sample liquid was transferred to a 5 mm NMR tube, followed by an addition of D_2O (325 μL) containing 3-(trimethylsilyl)-propionic-d$_4$ acid (1.5 mM) as an internal standard of known concentration. Compounds were identified from routine two-dimensional proton–proton and proton–carbon-correlated spectra. ^{1}H NMR spectra were collected using the zg automation program with the following parameters: 90° pulse angle, total relaxation delay of 13 s, and 32 scans at 300 K. In the ^{1}H NMR spectra, compounds are classified based on the functional groups present which also makes it possible to estimate the amounts of these compounds in the measured samples if the concentrations are at least on the mM level.

Samples for the 16S rRNA sequencing analysis were taken at the end of all the parallel cultivations with different carbon sources and at different COD/sulfate ratios. The bacterial cultures as well as whey and the conditioned sewage sludge were characterized by 16S rRNA sequencing at the Biocenter Oulu Sequencing Center as described previously in Virpiranta *et al.* (2019). The richness and alpha diversity of the bacterial species in the liquid cultivations were evaluated using species richness (S), the Shannon diversity index (H) described in Equation (1), where p_i is the proportion of the ith species, and true diversity, i.e., the effective number of the species (ENS), as described in Equation (2):

$$H = -\sum_{i=1}^{S} p_i \ln p_i \tag{1}$$

$$\mathrm{ENS} = e^{H} \tag{2}$$

RESULTS

Characterization of the low-cost carbon sources

The COD and pH values, as well as the total nitrogen and phosphorous values of all the low-cost carbon sources, are presented in Table 1. The whey had far higher COD and nutrient concentrations than the other two carbon sources. The peat solution had the lowest COD and nutrient concentrations, although autoclaving increased the COD significantly. Furthermore, the initial lactic acid and lactose concentrations of the whey were 1.28 ± 0.08 and 175 ± 15 g L^{-1}, respectively. However, it should be noted that the carbon sources also contained insoluble solids, which might have degraded during the bacterial cultivation and thus increased the COD and nutrient concentrations. The ash content of the dried peat was 7.6% ± 0.3% at 525 °C and 7.2% ± 0.3% at 950 °C. The elemental composition of the peat was determined to be as follows (average of three measurements $\pm$ standard deviation): C – 49.8% ± 0.2%, H – 5.8% ± 0.2%, and N – 2.6% ± 0.0%.

The dissolved organic compounds detected by NMR in synthetic mining water after the addition of carbon sources and cultivation of SRB consortium are listed in Table 2. In the succinate cultivations, all the succinate was totally consumed, and acetic acid was produced. Also, propionic acid was formed in cultivations at COD/sulfate ratios of 1.4 and 2.0. Sewage sludge contained acetic acid and propionic acid of which the latter was totally consumed during microbial growth. In addition, ethanol was produced in some of the parallel cultivations (thus the large deviation reported for the samples). The lactose in the whey cultivations had partly fermented into acetic acid, lactic acid, and propionic acid as well as into small amounts of ethanol and methanol. In the case of peat as a carbon source, the target organic compounds (mentioned in Table 2) were not accurately detected by NMR (as the concentrations were below the limit of detection), either in the sterile liquid or after microbial growth (data not shown). The NMR spectra of all parallel cultivations with each carbon source are presented in Supplementary Figures S1–S5.

Table 1 | COD, total dissolved nitrogen, total dissolved phosphorus, and pH values of the Kemicond-treated sewage sludge, peat solutions, and whey

Carbon source	COD (mg L^{-1})	Total dissolved N (mg L^{-1})	Total dissolved P (mg L^{-1})	pH
Sewage sludge	13,600 ± 200	75 ± 15	971 ± 3	6.45
Autoclaved peat solution (3.5 g L^{-1})	1,610 ± 20	59.3 ± 1.4	2.6 ± 0.3	5.75
Non-treated peat solution (3.5 g L^{-1})	320 ± 10	Not determined	Not determined	6.90
Whey	182,000 ± 4,000	2,900 ± 60	2,110 ± 50	5.45

Errors represent the range of two repeats.

Table 2 | Organic compounds dissolved from carbon sources before inoculation and produced by SRB consortia after 63 days of incubation in synthetic mining water at COD/sulfate ratios of 0.8, 1.4, and 2.0

Carbon source, COD/sulfate ratio	Acetic acid (mg L^{-1})	Lactic acid (mg L^{-1})	Propionic acid (mg L^{-1})	Succinic acid (mg L^{-1})	Ethanol (mg L^{-1})	Methanol (mg L^{-1})	Lactose (mg L^{-1})
Succinate 0.8	n.d.	n.d.	n.d.	1,900	n.d.	n.d.	n.d.
Succinate 0.8 SRB	1,160 ± 110	n.d.	n.d.	n.d.	2 ± 3	n.d.	n.d.
Succinate 1.4	n.d.	n.d.	n.d.	3,300	n.d.	n.d.	n.d.
Succinate 1.4 SRB	1,640 ± 20	n.d.	440 ± 50	n.d.	n.d.	n.d.	n.d.
Succinate 2.0	n.d.	n.d.	n.d.	4,700	n.d.	n.d.	n.d.
Succinate 2.0 SRB	1,660 ± 50	n.d.	1,480 ± 50	n.d.	n.d.	n.d.	n.d.
Sewage sludge 0.8	600	n.d.	200	n.d.	n.d.	n.d.	n.d.
Sewage sludge 0.8 SRB	300 ± 100	n.d.	5 ± 6	n.d.	200 ± 400	n.d.	n.d.
Sewage sludge 1.4	800	n.d.	400	n.d.	n.d.	n.d.	n.d.
Sewage sludge 1.4 SRB	840 ± 30	n.d.	19 ± 5	n.d.	1,000 ± 2,000	n.d.	n.d.
Sewage sludge 2.0	1,200	n.d.	600	n.d.	n.d.	n.d.	n.d.
Sewage sludge 2.0 SRB	1,240 ± 50	n.d.	n.d.	n.d.	400 ± 300	n.d.	n.d.
Whey 0.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	390
Whey 0.8 SRB	131 ± 13	220 ± 40	39 ± 14	n.d.	6.0 ± 0.5	8 ± 2	260 ± 110
Whey 1.4	n.d.	10	n.d.	n.d.	0.5	n.d.	650
Whey 1.4 SRB	140 ± 20	240 ± 30	60 ± 14	n.d.	14 ± 8	7.5 ± 1.5	370 ± 40
Whey 2.0	n.d.	140	n.d.	n.d.	33	n.d.	960
Whey 2.0 SRB	231 ± 1	420 ± 30	134 ± 7	n.d.	14.3 ± 1.3	19.9 ± 0.9	690 ± 30

Averaged values of three parallel cultivations with standard deviations are presented. Results of uninoculated waters are single repeats.
n.d., not detected.

Effect of the low-cost carbon sources on sulfate removal

The growth of SRB was visually observed as the formation of a black iron sulfide precipitate after 1 week of incubation at 6 °C with every carbon source. However, the most intense color change was observed in cultures grown with succinate. During the 63 days of incubation, sulfate concentrations decreased by 1,290–1,680, 670–1,090, 650–800, and 260–370 mg L^{-1} in cultures grown with succinate, sewage sludge, peat, and whey, respectively (Figure 1). Thus, there was no significant difference between the different COD/sulfate ratios.

With succinate as a carbon source, all the test series followed a similar trend during the first 28 days. After that, the sulfate reduction clearly slowed down at all the COD/sulfate ratios, but most at the ratio of 0.8. With sewage sludge as a carbon source, the most rapid sulfate reduction occurred during the first 20 days. After that, the reduction rates decreased
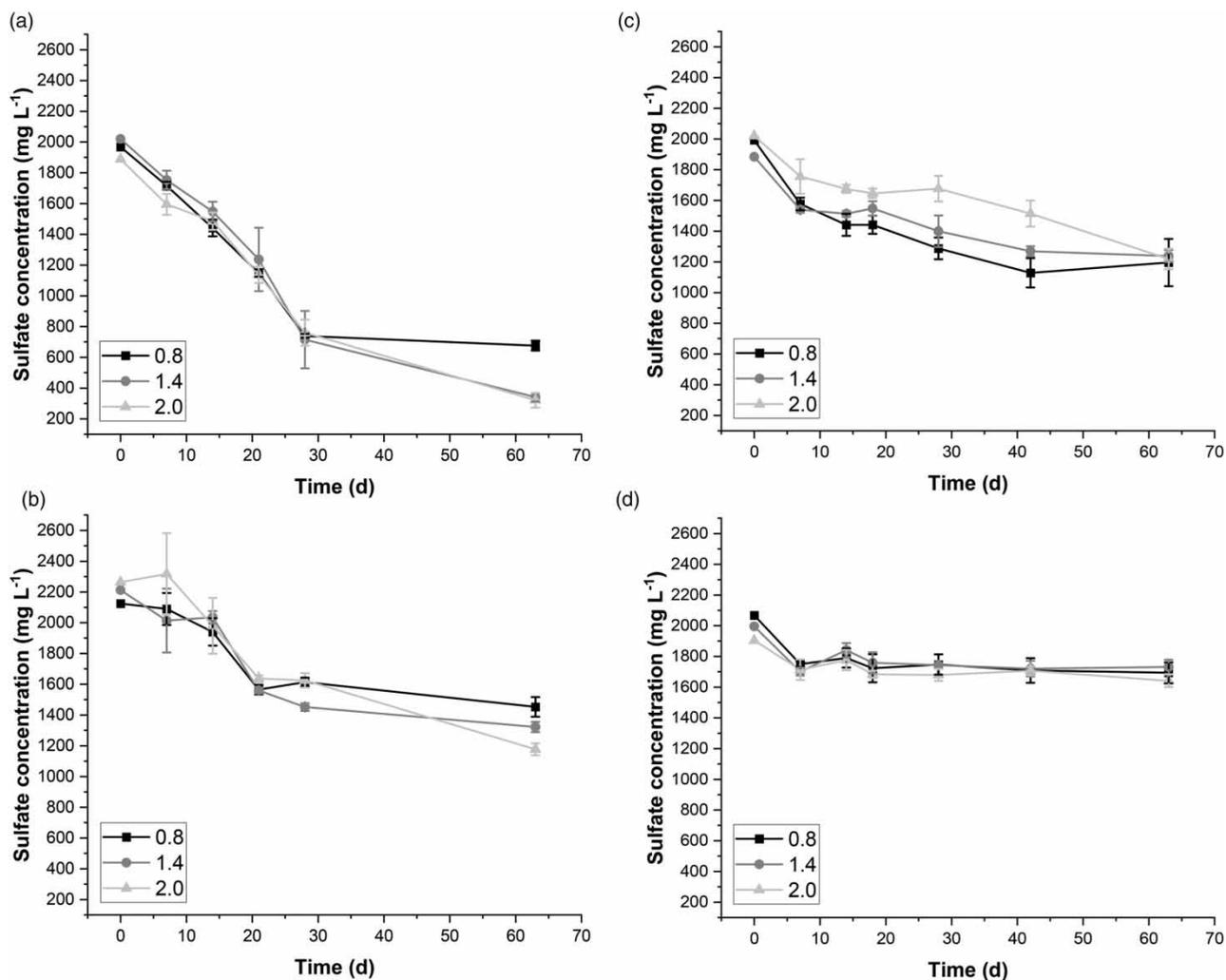
Figure 1 | Sulfate consumption during 63 days of incubation at 6 °C in synthetic mining water with different carbon sources: (a) succinate, (b) sewage sludge, (c) peat, and (d) whey. Symbols: COD/sulfate ratio 0.8 (square), 1.4 (circle), and 2.0 (triangle). Averaged values of three parallel cultivations with standard deviations are presented for each data series.

significantly, and the lowest sulfate concentration was achieved at the highest COD/sulfate ratio, and vice versa. With peat as a carbon source, the most rapid decrease in sulfate concentration occurred during the first week of cultivation. Subsequently, the sulfate reduction slowed slightly and the sulfate concentrations at the end were approximately on the same level at all the COD/sulfate ratios. With whey as a carbon source, sulfate reduction was rapid during the first week of incubation. However, after that reduction stopped almost completely and the differences between sulfate concentrations achieved at different COD/sulfate ratios were only minor.

Changes in the pH values throughout the cultivations are presented in Figure 2. There were already differences in the pH values between the mining waters supplemented with the carbon sources before inoculation, since some of the carbon sources lowered the pH more than others. With succinate, the pH was around 6.6 before inoculation, approximately 6.2 with sewage sludge, only around 5 with peat, and approximately 5.2 with whey. However, the pH increased for all the cultivations after inoculation and start of sulfate reduction. With succinate as a carbon source, the pH increased quickly to approximately 7.3 during the first 2 weeks and then started to stabilize. In the case of sewage sludge, the pH stayed relatively steady throughout the incubation. There were some minor variations in the pH values during the first 3 weeks of incubation. After that, the pH increased to approximately 6.5 at all of the COD/sulfate ratios in the sewage sludge cultivations. With peat as a carbon source, the pH increased quickly to approximately 6.1 during the first week of incubation, which correlated well with the rapid sulfate reduction at the beginning. After the first week, the pH values varied between 5.9 and 6.5.
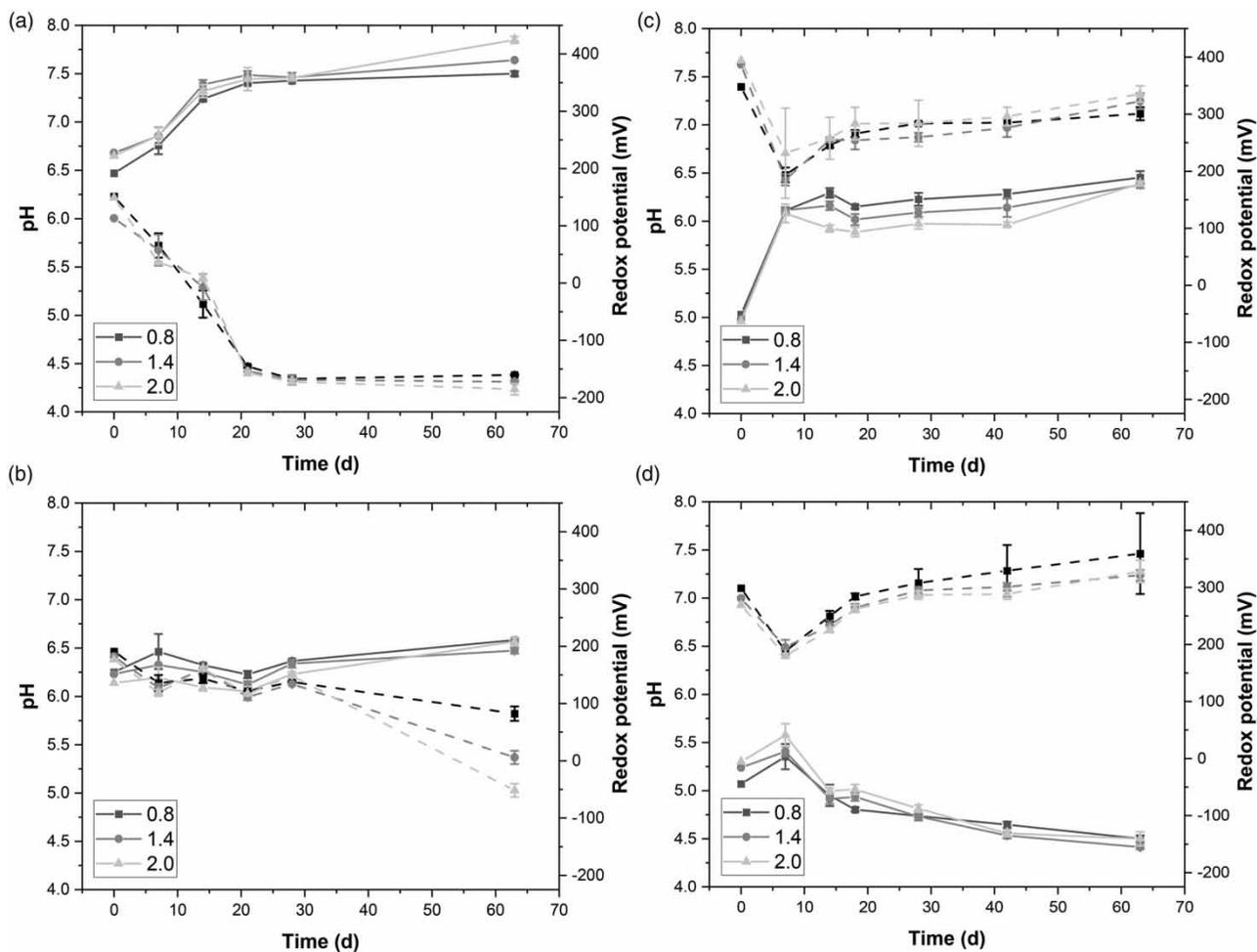
Figure 2 | Change in pH (solid line) and redox potential (dashed line) during 63 days of incubation at 6 °C in synthetic mining water with different carbon sources: (a) succinate, (b) sewage sludge, (c) peat, and (d) whey. Symbols: COD/sulfate ratio 0.8 (square), 1.4 (circle), and 2.0 (triangle). Averaged values of three parallel cultivations with standard deviations are presented for each data series.

Unexpectedly, in the case of whey, the pH started to decrease after 1 week of cultivation and eventually reached a level of 4.4–4.6. At the same time, the sulfate reduction also stopped.

Changes in the redox potentials correlated with the pH values with each carbon source throughout the cultivation (Figure 2): when the pH increased, the redox potential decreased and vice versa. With succinate as a carbon source, the average redox potential of all cultivations was 140 mV at the beginning. The value decreased to −152 mV during the first 3 weeks. After that, the redox potentials kept decreasing slowly, reaching a final value of −172 mV at the end of the cultivation period. However, in the case of the low-cost carbon sources, the redox potentials were positive almost all the time. Except for the highest COD/sulfate ratio of sewage sludge cultivations, where the average redox potential eventually reached −52 mV, the average redox potentials remained slightly positive (6–82 mV).

Sulfate adsorption by peat

The results of the adsorption tests with autoclaved and non-treated peat are presented in Table 3. No sulfate adsorption was detected during the 3-day experiment at the COD/sulfate ratio of 0.8 and the ratio of 1.4 for non-treated peat. However, at the ratio of 2.0 (highest peat dosage), sulfate was adsorbed slightly more with both autoclaved and non-treated peat and thus resulted in the highest sulfate uptake capacity. Furthermore, during the incubation at each COD/sulfate ratio, the pH of the mining water decreased to 6.1 ± 0.1 (average of all experiments ± standard deviation), and the redox potential increased significantly.

Table 3 | Sulfate uptake capacities of the autoclaved and non-treated peat, and sulfate removal rates in synthetic mining water (2 g L^{-1} sulfate) containing different amounts of peat and incubated at 6 °C for 3 days

COD/sulfate ratio	Peat dosage (g L^{-1})	Sulfate removal (%)	Sulfate uptake capacity (mg g^{-1})
0.8 autoclaved peat	3.5	0	0
0.8 non-treated peat	3.5	0	0
1.4 autoclaved peat	6.1	5	15
1.4 non-treated peat	6.1	0	0
2.0 autoclaved peat	8.7	10	19
2.0 non-treated peat	8.7	8	22

Effect of the low-cost carbon sources on bacterial distribution

All of the liquid cultures consisted mostly of three bacterial phyla – Proteobacteria, Firmicutes, and Bacteroidetes. Most of the SRB belonged to the genera *Desulfobulbus* and *Desulfovibrio*, which are Proteobacteria, although some sulfate-reducing Firmicutes belonging to the *Peptococcaceae* family were also present. The relative distribution of SRB in the liquid cultivations and in the original conditioned sewage sludge is presented in Figure 3. The relative abundance in the liquid cultivations of the most abundant bacteria (other than SRB) is shown in Figure 4. The SRB distribution expressed as the number of sequence reads is presented in Supplementary Figure S6. In the succinate cultivations, the average relative abundance of SRB varied from 5 to 11%. The highest value was detected when the amount of the carbon source was highest. In addition, the relative abundance of *Proteiniclasticum* sp. belonging to the *Clostridiaceae* family was significant in succinate cultivations at the COD/sulfate ratio of 2.0. The abundance was 3, 4, and 16% at ratios of 0.8, 1.4, and 2.0, respectively, and only 0–1% when low-cost carbon sources were used.

In peat cultivations, the average relative abundance of SRB was 8–9%. The samples at COD/sulfate ratios of 0.8 and 1.4 were dominated by the genus *Desulfobulbus*, whereas the sample at the highest COD/sulfate ratio was dominated by the *Desulfobulbaceae* family. Approximately 4% of the bacteria in the conditioned sewage sludge were SRB, being mainly *Desulfovibrio mexicanus* (Proteobacteria) and *Desulfosporosinus meridiei* (Firmicutes), which were also present in the liquid
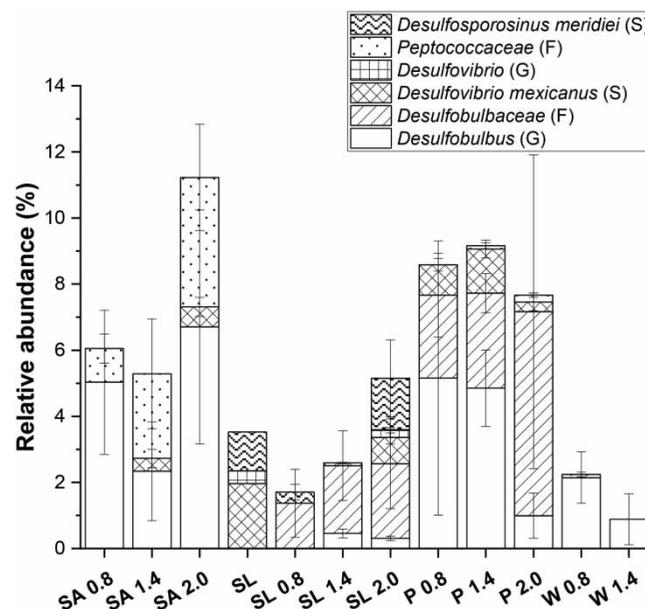
Figure 3 | Distribution of SRB in the original conditioned sewage sludge and after 63 days of incubation at 6 °C in synthetic mining water with different carbon sources and COD/sulfate ratios (0.8, 1.4, and 2.0). Abbreviations: bacterial species (S), bacterial family (F), bacterial genus (G), succinate (SA), sewage sludge (SL), peat (P), and whey (W). Averaged values of three parallel cultivations with standard deviations are presented for each data series, except for the original conditioned sewage sludge, which is the result of a single repeat.
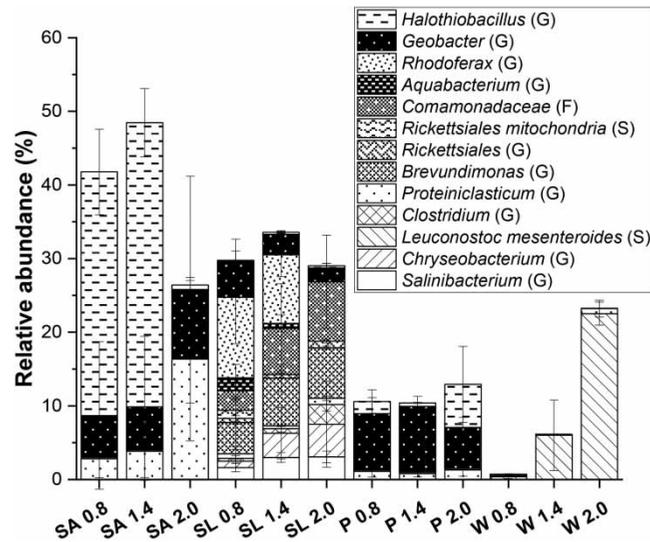
Figure 4 | Relative abundance of the most abundant bacteria (other than SRB) after 63 days of incubation at 6 °C in synthetic mining water with different carbon sources and COD/sulfate ratios (0.8, 1.4, and 2.0). Abbreviations: bacterial species (S), bacterial family (F), bacterial genus (G), succinate (SA), sewage sludge (SL), peat (P), and whey (W). Averaged values of three parallel cultivations with standard deviations are presented for each data series.

cultivations containing the sludge. The relative abundance of SRB in the sludge cultivations was 2–5%; the abundance being higher with a larger amount of sludge. Other significantly abundant bacterial genera detected in each sludge cultivation, but not with other carbon sources, were *Salinibacterium* sp. (Actinobacteria) (2–3%), *Chryseobacterium* sp. (Bacteroidetes) (1–4%), *Brevundimonas* sp. (Proteobacteria) (4–7%), and the *Comamonadaceae* family (including *Rhodoferax* sp.) (Proteobacteria) (8–16%). These genera were not dominant in the original conditioned sludge. The sewage sludge contained approximately 8.5% of possibly pathogenic bacteria belonging to the genera *Clostridium* (including *C. subterminale* and *C. celatum*) and *Rickettsiales*. The whey contained only one bacterial species, the lactic acid bacterium *Leuconostoc mesenteroides*, which also thrived in the liquid cultivations with the highest whey content. The relative abundance of *L. mesenteroides* was 23% of all the observed bacterial species at the COD/sulfate ratio of 2.0, and 6% at the COD/sulfate ratio of 1.4, but near zero at the COD/sulfate ratio of 0.8. The relative abundance of SRB in whey cultivations varied from 1 to 2% and the highest abundance was observed in the sample at the COD/sulfate ratio of 0.8.

Other bacteria present in the cultivations which may influence the sulfur cycle were *Geobacter* and *Halothiobacillus*. The genus *Geobacter* was present in all cultivations, except in those with whey as a carbon source. The relative abundance varied from 6 to 9% with both succinate and sewage sludge, and from 2 to 5% with peat as a carbon source. The genus *Halothiobacillus* was mainly found in succinate cultivations with a lower substrate content. The relative abundance was 33, 39, and 1% at COD/sulfate ratios of 0.8, 1.4, and 2.0, respectively.

Sewage sludge resulted in the highest microbial richness and diversity in the liquid cultures – over one hundred bacterial species were found – and the Shannon diversity index as well as ENS were the highest (see Table 4). The species richness was

Table 4 | Species richness (*S*), Shannon diversity index (*H*), and effective number of the species (ENS) in liquid cultivations with succinate, sewage sludge, peat, or whey as a carbon source after 63 days of incubation at 6 °C

Carbon source	S	H	ENS
Succinate	31 ± 4	2.05 ± 0.11	7.8 ± 1.2
Sewage sludge	110 ± 20	3.3 ± 0.2	28.0 ± 1.3
Peat	42 ± 5	2.5 ± 0.2	12.7 ± 1.2
Whey	15 ± 4	1.24 ± 0.11	3.4 ± 1.2

Averaged values of nine cultivations (three different COD/sulfate ratios and three replicates for each ratio) with standard deviations are presented.

significantly larger when the amount of sludge was the highest. With whey, succinate, and peat as the carbon sources, there was little variance in species richness between the different COD/sulfate ratios. However, in the Shannon index values, there was no clear difference between the COD/sulfate ratios. The species richness at different sequencing depths is presented in Supplementary Figure S7.

DISCUSSION

The reference carbon source succinate was the most efficient source of carbon, as expected, since the SRB were originally enriched with succinate. The largest decrease in sulfate concentration was observed at the COD/sulfate ratio of 1.4 (sulfate removal rate 83%). Higher COD/sulfate ratios of 1.5–2.25 have been suggested for efficient sulfate reduction by El bayoumy et al. (1999), Velasco et al. (2008), and Barbosa et al. (2014). However, the COD/sulfate ratio of 0.8 was also found to be feasible (sulfate removal rate 66%) in this study. There were no significant differences in the sulfate reduction rates between the different COD/sulfate ratios during the first month of incubation. According to the NMR results, all of the succinate was utilized in the cultivations regardless of the COD/sulfate ratio. At ratios of 0.8 and 1.4, the acetic acid amount produced was in accordance with the reaction stoichiometry (Equation (3)), whereas at the ratio of 2.0, the acetic acid concentration would have been greater if all of the succinate had been utilized for sulfate reduction.

$$4(CH_2)_2(COO^-)_2 + 3\,SO_4^{2-} + 2.5H^+ + 2H_2O \rightarrow 4CH_3COO^- + 6\,HCO_3^- + 1.5H_2S + 1.5HS^- + 2CO_2 \qquad (3)$$
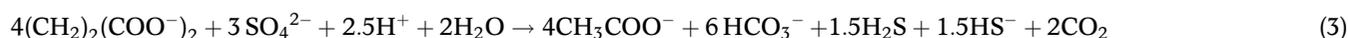
Furthermore, a large amount of propionic acid was formed in succinate cultivations at the COD/sulfate ratio of 2.0. *Desulfobulbus propionicus* is known to degrade propionate in the presence of sulfate and produce it in the absence of sulfate (Kremer & Hansen 1988). The latter situation could have occurred when the sulfate concentration was diminished at the end of the cultivation. Another possibility is that propionate was produced by other fermenting organisms present, e.g., *Proteiniclasticum* sp., and was further utilized by SRB. *Proteiniclasticum* sp. is known to produce propionate as a fermentation product (Zhang et al. 2010), and it was present in a relatively high amount (16%) when the COD/sulfate ratio of 2.0 was used. Consequently, at higher succinate concentrations, a larger amount of the substrate was consumed for purposes other than sulfate reduction, which is an undesirable effect. The same phenomenon was observed by Zhao et al. (2008). Thus, it is important to optimize the amount of substrate according to the desired level of sulfate removal. One reason for the sulfate reduction decrease in batch cultivations may be sulfide inhibition (Hao 2003). For example, Okabe et al. (1992) observed a 50% inhibition in sulfate reduction when the total sulfide concentration was approximately 500 mg L^{-1}. Therefore, increasing the substrate amount in batch operations may not necessarily increase sulfate removal. However, in this study, the largest relative abundance of SRB (11%) was detected at the highest succinate concentration. At the COD/sulfate ratio of 1.4, the abundance of SRB was only 5%. In addition, the bacterial diversity was lower in succinate cultivations as compared with peat and sludge cultivations (see Table 4). Since the sulfate reduction reaction releases bicarbonates, an increase in pH during the cultivations is to be expected. Both the pH and redox potential stabilized after 1 month of cultivation with succinate when the sulfate reduction slowed down.

The slightly lower initial pH in sewage sludge cultivations compared with succinate cultivations was probably due to the Kemicond sludge conditioning process, where sewage sludge is treated by sulfuric acid and hydrogen peroxide and the pH is adjusted to 3–5 (Cornel et al. 2004; Schaum et al. 2006). Interestingly, there was a drop in pH during the most rapid sulfate reduction stage, and the pH started to increase only after the sulfate reduction had slowed down. In addition, the redox potential started to decrease noticeably at the same time. The decrease in pH was probably caused by the acetic acid produced in the incomplete oxidation of organic carbon by SRB, and the later increase in pH was due to the utilization of acetate by other microbes. According to the NMR results, acetic acid concentration did not increase because of sulfate reduction, and even decreased in the case of the COD/sulfate ratio of 0.8 (see Table 2 in the Results section), which indicates the presence of acetate-utilizing microbes. *Salinibacterium* sp., *Chryseobacterium* sp., *Brevundimonas* sp., and *Rhodoferax* sp., all of which are known to utilize acetate (Finneran et al. 2003; Ryu et al. 2007; Wen et al. 2016; Nazina et al. 2020), were detected with relatively high abundances in the sludge cultivations. The genus *Rhodoferax* was especially dominant. However, none of these genera were dominant in the original sludge, and thus they were activated by the acetate produced in sulfate reduction. The SRB present (*Desulfobulbus* sp., *Desulfovibrio mexicanus*, and *Desulfosporosinus meridiei*) are all incomplete oxidizers and thus do not utilize acetic acid (Widdel & Pfennig 1982; Hernandez-Eugenio et al. 2000; Robertson et al. 2001). The NMR results indicate the utilization of propionic acid as a carbon source in sulfate reduction.

Even though the sewage sludge was disinfected by the Kemicond process, it still contained small amounts of possibly pathogenic bacteria, and the addition of sludge increased the bacterial diversity in the SRB cultivations significantly; the species richness and the ENS were high in the sewage sludge cultivations compared with the other carbon sources (Table 4). For industrial applications, high bacterial diversity is a benefit, since it prevents contamination of the process and supports the stability of the consortium (White *et al.* 1998; Boothman *et al.* 2006). However, in this case, it cannot be clearly stated whether the good performance of the SRB was due to high bacterial diversity or the easily available carbon source. The amount of SRB was proportional to the sulfate removal efficiency in the sludge cultivations. The average sulfate removal rate varied from 32 to 48%, whereas Liu *et al.* (2018) reported removal rates of 79–92% with COD/sulfate ratios of 1.0–2.0 in a continuously operated bioreactor using sewage sludge as a carbon source. In addition, Ristow *et al.* (2004) achieved sulfate removal rates of 73.5–96.6% with COD/sulfate ratios of 1.0–2.65 in fed batch experiments using sewage sludge as a carbon source. Fed batch cultivations were also tested by Peng *et al.* (2017) with a sludge content of 10–30% in the feed, resulting in sulfate removal rates of 38–71%. In the sludge used by Peng *et al.* (2017), only 0.59% of the bacteria were sulfate reducers; however, as much as 27.4% of the bacterial community in the reactor liquid consisted of SRB, most of them belonging to the *Desulfobulbaceae* and *Desulfobacteraceae* families. Even though the proportion of SRB in this study was small compared with the study of Peng *et al.* (2017), the difference between sulfate removal rates was insignificant.

Most of the organic material in peat is formed of humic substances: humin, humic acid, and fulvic acid (Klavins & Purmalis 2013), of which the last two can readily serve as carbon and nitrogen sources for microbes. In this study, peat worked well as a nutrient source, since over 40 different bacterial species were found in the peat cultivations, of which approximately 13 were equally abundant species (Table 4). The relative abundance of SRB, as well as sulfate removal rates (34–40%), were similar in all of the peat cultivations at different COD/sulfate ratios. The sulfate removal rate with peat was higher than that in the results of the study by Nielsen *et al.* (2019), where the removal rate remained at 13.8%. In cultivations with peat as a carbon source, the decrease in sulfate concentration was mainly caused by biological sulfate reduction. However, adsorption of the sulfate by peat was also detected. The sulfate adsorption was clearly observed with the highest peat amount and autoclaving of the peat solution did not increase the sulfate removal capacity (Table 3). However, at the COD/sulfate ratio of 1.4, the adsorption of sulfate did not occur with the non-autoclaved peat solution. According to DeLaune & Reddy (2005), sulfate reduction occurs when the redox potential is between −100 and −200 mV. Thus, most of the biological sulfate reduction probably occurred at the beginning of the cultivation with peat, when the redox potential decreased. However, it never reached negative values. Nielsen *et al.* (2019) detected an increase in redox potential values when less organic carbon was available and the activity of SRB decreased. The simultaneous increase in pH at the beginning of the cultivations also supports this hypothesis (Figure 2(c)). Initially, the pH of the mining water was much lower with peat than with other carbon sources, and the pH also dropped during the incubation without bacterial inoculum. The same effect of peat on the pH was detected by Nielsen *et al.* (2019). It is likely that the autoclaving of the mining water with peat increased the dissolution of the humic and fulvic acids from peat into the water, since the COD of the autoclaved peat solution was significantly higher than the COD of the non-treated peat solution (Table 1). However, in NMR analysis, no significant concentrations of any target organic compounds were detected, either in the uninoculated water containing peat or after microbial growth.

The whey used in this study contained mostly lactose and only a small amount of lactic acid. While lactic acid is usually the most favorable carbon source for SRB, some lactose-utilizing SRB consortia have also been enriched (Wolicka 2008; Wolicka & Borkowski 2009). Sass *et al.* (2002) also reported the characterization of a lactose-utilizing *Desulfobulbus mediterraneus* sp. However, the SRB exploited in the present study were probably not able to utilize lactose as they were originally enriched using succinate as a carbon source. The experiment resulted in a sulfate removal rate of only 13–18%. Higher removal rates were achieved with whey as a carbon source by Wolicka & Borkowski (2009) in batch experiments and by Jiménez *et al.* (2010) in a continuous bioreactor, namely 55 and 68%, respectively. After 1 week of incubation, sulfate reduction stopped, the redox potential started to increase and the pH to decrease, indicating that fermentative microorganisms were favored (Farhadi *et al.* 2013). Conversion of lactose to lactic acid in all of the whey cultivations was detected by NMR analysis (Table 2). The lactic acid bacterium *L. mesenteroides* found in the whey is known to ferment lactose into lactic acid (Özcan *et al.* 2019), which could further act as a carbon source for SRB. However, in this case, the lactic acid production probably made the pH too low for SRB and many other bacteria to survive: only around three bacterial species were found to be equally abundant in the whey cultivations (Table 4). In addition, the formation of acetate, propionate, and ethanol in whey cultivations can be explained by the metabolism of *L. mesenteroides* (Özcan *et al.* 2019; Yang *et al.* 2020).

This study focused on the biological sulfate removal from synthetic mining wastewater in the absence of metals. However, in a real mine water treatment application, removal of metals present in mining waters should also be considered. The metals in mining waters and their concentrations differ greatly from mine to mine. Many divalent and monovalent metals can be precipitated as metal sulfides with the H_2S generated in the biological sulfate reduction process. The inhibitory effect of toxic metals on SRB can be avoided by the H_2S precipitation of transition metals prior to the sulfate reduction step (Huisman *et al.* 2006). In addition, biological sulfate removal can be integrated with other treatment methods. For example, if the sulfate concentration is very high, gypsum precipitation can be used for the removal of most of the sulfate, residual metals can be removed by adsorption and biological sulfate reduction can be used as a second polishing step to achieve lower sulfate concentrations (Hurtado *et al.* 2018).

CONCLUSION

With a truly abundant and low-cost carbon source, namely conditioned sewage sludge, an encouraging sulfate removal rate was achieved even at low temperature. During the first 3 weeks of incubation, the removal rate was quite similar to that achieved with the reference carbon source, succinate. The propionic acid present in the sludge was utilized as a carbon source by SRB. Peat also performed well in terms of sulfate reduction. We proved the potential of using peat simultaneously as a biosorbent to adsorb sulfate and as a carbon source for biological sulfate reduction. In whey cultivations, lactic acid was successfully produced from lactose, but not further utilized by the SRB. Succinate worked efficiently as a carbon source for the SRB; the sulfate reduction and changes in pH and redox potential were highly predictable throughout the bacterial growth. The highest tested COD/sulfate ratio of 2.0 did not increase sulfate reduction when compared with the ratio of 1.4. In further studies, low-temperature sulfate reduction will be tested with real mining-impacted waters in a continuously operated bioreactor.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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