Compositions of the Microbial Consortia Present in Biological Sulphate Reduction Processes During Mine Effluent Treatment

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Abstract The microbial consortia in sulphate-reducing bioreactors with different operating conditions were studied and compared to the sulphate reduction efficiencies. The results showed vast differences in microbial communities among the reactors. The fraction of sulphate-reducing bacteria correlated with the bioreactor performance. This study sheds new light to the biological sulphate-reducing process applied in bioreactors, which is traditionally seen as a black box.

Key words sulphate-reducing bacteria, bioreactor, sulphate removal, microbial communities, mine water treatment

Introduction

In biological sulphate reduction, sulphate is converted to sulphide in anaerobic conditions by sulphate-reducing bacteria (SRB) that utilize an external carbon source and electron donor. Simultaneously, alkalinity is produced in the form of bicarbonate (Eq. 1) (Vestola and Mroueh 2008).

$$2\,CH_2O + SO_4^{2-} \rightarrow H_2S + 2\,HCO_3^- \quad \text{(Eq. 1)}$$

This process enables the treatment of acidic, sulphate-containing waste water streams. These features are typical to waste waters of the mining industry, and substantial research and development are conducted in the biological treatment of such mining effluents (Bijmans et al. 2011).

Most of the SRB belong to the class Deltaproteobacteria, including genera such as *Desulfovibrio*, *Desulfobulbus* and *Desulfomicrobium*, with some representatives in other groups, e.g. phylum Nitrospirae and the Firmicutes class Clostridia (Muyzer and Alfons 2008). Most known SRB are mesophiles, and the highest sulphate reduction efficiencies in bioreactors are usually obtained in the temperature range of 30 – 45 °C (Bijmans et al. 2011). A pH range of 7.0 – 8.0 in the bioreactor is considered optimal for SRB (Moosa and Harrison 2006), although efficient sulphate removal has also been obtained at lower pH of 4.0 – 5.0 (Santos and Johnson 2017). However, a minimum redox potential of at least -150 mV is required for biological sulphate reduction to occur (Barton 1995).

Substrates used for biological sulphate reduction can be simple compounds, such as hydrogen or lactate, or more complex organic waste materials, such as woodchips or manure. The advantages of simple substrates include wide suitability for SRB, good availability and ease of dosing, whereas organic wastes can be a lower cost and more sustainable option (Bijmans et al. 2011). Full utilization of complex substrates requires co-operation among different microbial groups, and the microbial communities present in bioreactors operated with complex substrates can be expected to be more diverse than in bioreactors operated solely with simple substrates (Hiibel et al. 2011).

In this study, the microbial communities of the effluents of four different laboratory scale sulphate-reducing bioreactors were compared. The substrates used were either complex compounds (woodchips, hay, cow manure) or a combination of complex and simple (lactate, crude glycerol) compounds. Three of the bioreactors were operated in South Africa (SA) and one was operated in Finland (FIN). The effect of reactor configuration and substrate on microbial communities and subsequent sulphate reduction performance is discussed.

Methods

Bioreactors and sampling. The biological systems in this work included two down-flow anaerobic flooded reactors (T1, T2), a continuous stirred-tank reactor (CSTR) (T3) and an up-flow anaerobic sludge blanket (UASB) reactor (T4) (Tab. 1). The bioreactors were operated with either only complex (T1), or a combination of complex and simple substrates (T2, T3, T4). The operating temperatures ranged from 21°C to 30°C while the influent sulphate concentrations ranged from 1.1 g L^{-1} to 4.5 g L^{-1}. In addition, T2 and T3 received ammonium and phosphate added to the feed. Hydraulic retention times (HRT) varied from one day to 21 days (Tab. 1). Samples for both chemical and microbial analyses were taken from bioreactor effluents after achieving steady operation (after 100 – 300 days of operation). The results of chemical measurements as well as the main microbial findings in the bioreactor effluents are included in Tab. 1.

T1 and T2 were inoculated with cow manure, and T3 with a SRB culture maintained at Mintek, South Africa. Bioreactor T4 was inoculated with the Mintek SRB culture and fresh cow manure, which also served as the sludge blanket for microbes.

T1 simulated a passive system packed with woodchips, wood shavings, hay and manure in a 40/20/20/20 ratio, whereas T2 and T3 contained woodchips and were fed waste glycerol obtained from the biofuel industry (5 ml L^{-1}). T4 was fed with cow manure and lactate in a mass ratio of 75/25 based on the carbon content, with a total substrate excess of 50% for biological sulphate reduction. It was assumed that one mole of sulphate (96 g/mol) requires two moles of carbon (12 g/mol) for biological reduction (Eq. 1), and thus the required organic carbon is one quarter of the sulphate to be reduced. Substrate mixture was added to T4 periodically, with a sufficient substrate dose every 3 – 4 days, as a continuous dosing of manure was not technically possible.

Chemical analyses. Effluent pH and redox potential levels in T1, T2 and T3 were measured with a Metrohm pH sensor and Hamilton redox sensor (mV, vs Ag/AgCl). In T4, pH and

***Table 1**. The operating conditions, influent specifics, location and results of the effluent analyses of the bioreactors in this work.*

	T1	T2	T3	T4
Bioreactor type	Down-flow anaerobic flooded column	Down-flow anaerobic flooded column	CSTR	UASB
Operating T (°C)	23	24	30	21
Substrate	Woodchips, hay, manure	Woodchips, crude glycerol	Woodchips, crude glycerol	Manure, lactate
Added nutrients	None	1.2g/L $(NH_4)_2SO_4$, 0.4g/L H_3PO_4	1.2g/L $(NH_4)_2SO_4$, 0.4g/L H_3PO_4	None
Influent sulphate (g L^{-1})	2.7	4.5	4.5	1.1
HRT (d)	21	9	4	1
Location (SA/ FIN)	SA	SA	SA	FIN
pH	8.04	7.05	7.62	7.40
Redox potential (mV)	-236	-399	-396	-176
Relative sulphate removal (%)	95	83	82	59
Total sulphate removal rate (mg L^{-1} d^{-1})	122	415	923	572
Relative abundance of SRB (%)	1.1	5.4	10.2	8.7

redox potentials were measured with a Consort multi-parameter analyser C3040 equipped with Van London-pHoenix Co. electrodes (Ag/AgCl in 3M KCl). Sulphate concentrations were analysed with the barium sulphate method (Clesceri et al. 1998).

Microbial analyses. The microbial communities in the effluents of the four different bioreactors were characterized with high throughput amplicon sequencing targeting the prokaryotic 16S rRNA gene. The primers used were Bact_0341F/Bact_805R (Herlemann et al. 2011; Klindworth et al. 2013), targeting the variable region V3-V4 of the 16S rRNA gene. For T4, amplicons were prepared for sequencing on the Iontorrent PGM platform from the forward primer, and T1, T2 and T3 were paired-end sequenced on the Illumina MiSeq platform. The Iontorrent sequences were trimmed and quality checked as described in Rajala et al. (2016). The MiSeq sequences were paired using the default quality score values assigned in QIIME version 1.9 (Caporaso et al. 2010).

The sequence data were subsequently analysed with the QIIME software, chimeric sequence reads were removed from the dataset with the USEARCH-algorithm (Edgar 2010) by *de*

novo detection and through similarity searches against the Greengenes reference dataset (Version gg_13_8) (DeSantis et al. 2006). Sequence reads were grouped in to Operational Taxonomic Units (OTUs) at minimum 97% sequence homology using the open OTU picking method in QIIME. Taxonomic assignments for the OTUs were based on the Greengenes (gg_13_8) reference database.

Results

Chemistry. The pH of the effluent was similar in all bioreactors, but the redox potentials were significantly lower in T2 and T3 than in T1 and T4 (Tab. 1). T1, T2 and T3 had higher relative sulphate removal efficiencies compared to T4, but according to the total sulphate removal rates, T3 had the highest sulphate removal, followed by T4, T2 and T1 (Tab. 1).

Microbiology. The number of prokaryotic 16S rRNA gene sequences obtained from the different bioreactors varied between 6996 reads for T3 and 39727 reads from T4.

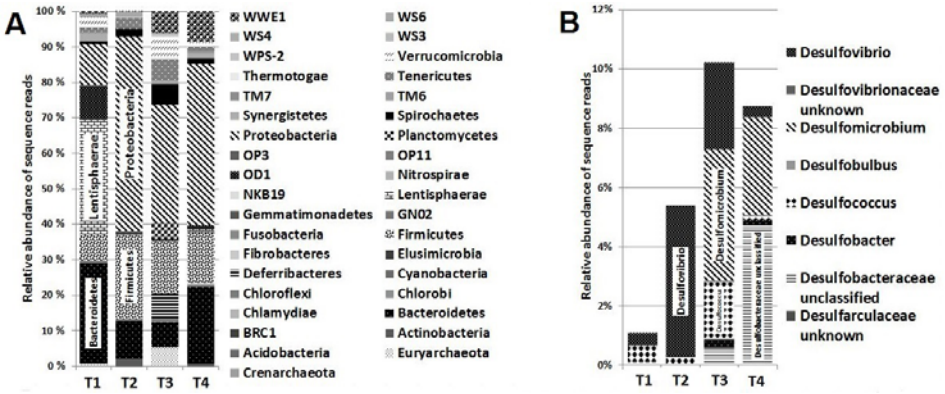

Figure 1 *A) The relative abundances of prokaryotic Phyla observed in bioreactor effluents based on the high throughput sequencing, and B) the relative abundances of SRB genera in the bioreactor effluents in detail.*

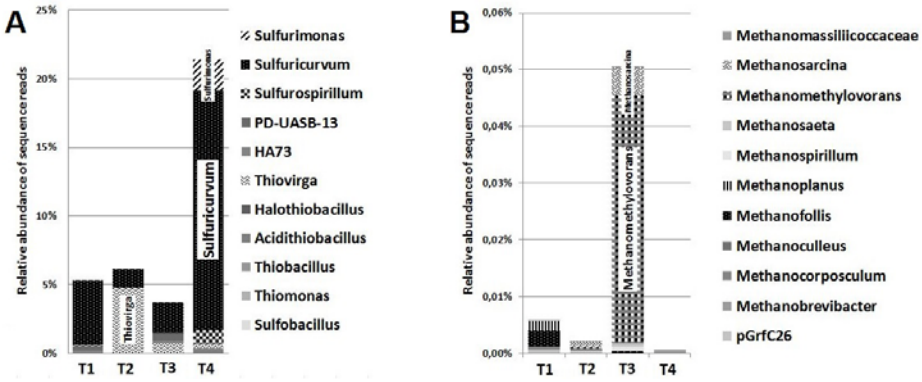

Figure 2 *A) The relative abundances of sulfur-oxidizing bacterial genera, and B) the relative abundances of archaeal genera in the bioreactor effluents.*

The majority of the microbial community in T1 consisted of Bacteroidetes, Clostridia, Lentisphaera and OD1 bacteria (Fig. 1A). In T2, the most abundant bacteria belonged to Firmicutes and Proteobacteria, and in T3 to Planctomycetes, Proteobacteria, Tenericutes, Verrucomicrobia and WWE1 bacteria. T4 had a high abundance of Proteobacteria, but especially Bacteroidetes, Firmicutes and WWE1 bacteria were abundant. In these samples, the sulphate reducers mostly belonged to the class Deltaproteobacteria (Fig. 1B). Deltaproteobacterial SRB were abundant in T2, T3 and T4. In T2, SRB belonging to the order Desulfovibrionales contributed with 5.1 % of the whole microbial community. In T3, the deltaproteobacterial SRB community consisted of the orders Desulfobacterales, Desulfovibrionales and Desulfuromonadales, contributing with 2.9 %, 7.4 % and 5.5 % of the total number of microbial sequence reads in the sample. In T4, the SRB community mainly consisted of Desulfobacterales and Desulfovibrionales, contributed with 5.1 % and 3.6 % of the microbial community. SRB belonging to the Firmicutes phylum (order Clostridiales) were not detected (Fig. 1B). Instead, T2 had a high abundance of bacteria belonging to the order Syntrophomionadaceae (10.5 % of the total sequence reads). Sulphide-oxidizing Epsilonproteobacteria were abundant in T4 (*Sulfurospirillum* 1 %, *Sulfuricurvum* 17.4 %, *Sulfurimonas* 2.3 %) in T4 (Fig. 2A). *Sulfuricurvum* was also abundant in T1, T2 and T3 (4.7 %, 1.4 % and 2.2 %, respectively). The archaeal abundance detected with the primers used was generally low, with the exception of T3, for which 5.1% of the obtained sequence reads belonged to metanogenic Archaea of the genera *Methanomethylovorans* (4.5 %) and *Methanosarcina* (0.5 %)(Fig. 2B).

Discussion

All of the tested bioreactors achieved functional sulphate reduction. Although relative sulphate removal was the highest in T1, long HRT caused the total sulphate removal rate to be the lowest of all tested bioreactors. The total sulphate removal rate increased in the experiments as HRT decreased, but in T4 the HRT was most probably too short for efficient sulphate removal. T3 had the best conditions for efficient sulphate removal: sufficient HRT, the highest tested temperature, a suitable mixture of simple and complex substrates and a reactor configuration enabling an effective contact between substrates and bacteria.

The bioreactor effluents contained different microbial consortia. In T1, the most abundant bacteria belonged to the Lentisphaera, Bacteroidetes and OD1 phyla. These bacterial groups are heterotrophic fermenters (Bauer et al. 2006; Choi et al. 2012; Wrighton et al. 2012). In addition, some Bacteroidetes have been shown to have a wide variety of hydrolytic enzymes with which they can degrade high molecular weight organic matter, such as plant polysaccharides, and the OD1 bacteria reduce sulphur. In the other bioreactors, Proteobacteria were the most abundant bacterial groups. SRB did not form the most abundant microbial group in any of the bioreactors. However, in T3 and T4 the relative abundance of SRB reached 10.2 % and 8.7 %, respectively (Tab. 1), whereas in the other bioreactors their relative abundance stayed below 5.5 %. The total sulphate removal rates went according to the order of SRB fractions, as higher SRB fraction resulted in a more removed sulphate. Interestingly, the relative abundance of sulphide-oxidizing bacteria was high, especially in T4, where their relative abundance was over 21 % (Fig. 2A). The abundant sulphur oxidizing

Epsilonproteobacteria in T4 might have oxidized the sulphide produced by the SRB, converting it back to sulphate, and thus decreasing the sulphate removal efficiency. The reason for the abundant sulphide oxidizers is not clear, but it may be an effect of the shorter HRT and lower operating temperature of T4 compared to the other bioreactors.

SRB generally utilize simple substrates more efficiently than complex organic matter, which usually contains slowly degradable compounds that require a long retention time in continuously operated bioreactors for efficient sulphate removal (Gibert et al. 2004). In this study, the effect of substrate on sulphate removal efficiency was difficult to differentiate, as other factors, such as HRT, had a greater influence on the bioreactor performance. T1 received only complex substrates, but the HRT was enough for an efficient relative sulphate removal. However, long HRT may have caused the depletion of sulphate early in the bioreactor, resulting in a decrease in the total fraction of SRB and an increase in the abundance of fermenting bacteria.

T4 had the lowest sulphate load, highest redox potential, lowest operating temperature, shortest HRT and no woodchips as carrier material for biofilm formation and long-term storage of carbon source. The presence of decaying woodchips may provide a steady source of small carbon compounds feeding the microbial consortia in the other bioreactors. Thus intervals of pulses of high concentrations of substrate and times of starvation may be avoided. This may produce a more stable sulphate-reducing consortium than when the bioreactor is fed at intervals of a few days. All SA bioreactors were operated at longer HRT than what was used with T4. The reason for short HRT used in T4 was the effort to optimize an application that would be as efficient as possible, so that the circulation of water in the mine would be swift. A fast turnover would be preferred, because it might not be feasible to store large amounts of water in the mine, although longer HRT could provide a high relative sulphate removal, as seen in T1. Whether the aim is to achieve a certain sulphate concentration in the effluent or a high total sulphate removal rate, the HRT among other parameters can be adjusted accordingly.

Based on these results, the fraction of SRB is a good indicator for sulphate-reducing bioreactor performance. However, the bioreactors of this study were so different that thorough comparison is difficult, as each bioreactor developed a unique microbial consortium over time, and a detailed analysis of the interactions is difficult to conduct. The relationship between microbiology and reactor performance requires more research. For example, experiments on identical bioreactors with varying operation parameters (such as temperature or HRT) should be conducted to suggest appropriate measures, for example, for decreasing the fraction of unwanted microbial groups and increasing the fraction of SRB. In addition, samples from the sludge blanket could help to characterize the microbial population in the bioreactor more accurately.

Conclusions

We found significant differences in the microbial community composition in the different bioreactor effluents based on the high throughput sequence analysis. In the bioreactor with

the highest total sulphate removal rate, the highest relative abundance of SRB was detected. In addition, we showed that the general microbial community composition of the bioreactor with the longest HRT differed significantly from the other bioreactors. Characterizing the microbial communities in detail gives us a tool to follow the development of the microbial consortia in the bioreactors and obtain information about what factors are especially important for the development of a well performing bioreactor. It is a more sophisticated tool than the 'trial and error' approach when altering bioreactor configuration or operation parameters for enhancing the sulphate removal efficiency. With more research, even single methods for removing specific groups and enhancing others could be identified, which would greatly assist in improving sulphate-reducing bioreactor performance universally, regardless of the system configuration in question.

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