Research Article

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Electric discharge by sulphide shuttling bacteria

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Abstract

BACKGROUND: In biodesulphurisation processes, sulphide oxidising bacteria (SOB) convert toxic sulphide to sulphur. Haloalkalophilic SOB are known to anaerobically remove sulphide from solution and release electrons when subsequently exposed to an electrode. This makes SOB able to spatially decouple sulphide removal and current production, thereby acting as sulphide shuttles. Little is known about the kinetics of electron release by sulphide shuttling SOB. To gain more insight into the sulphide shuttling mechanism and electron release, current production of abiotic sulphide and sulphide shuttling SOB was compared.

RESULTS: SOB communities dominated by *Thioalkalivibrio sulfidophilus* were incubated with sulphide in batch experiments. After sulphide was removed, SOB were discharged in an electrochemical cell. Anode potential, sulphide load and biomass concentration were varied. Current was produced at potentials of −0.2 V *versus* Ag/AgCl and higher, and at a potential of 0.1 V a coulombic efficiency of 70% was reached in 30 min. The maximum charge recovered from biologically stored sulphide was 4900 ± 810 mC mgN^{-1}. Discharge kinetics were not affected by biomass concentration or degree of sulphide removal and kinetics of biotic and abiotic current production were similar.

CONCLUSION: Current production of sulphide shuttling SOB and abiotic sulphide was highly similar. This implies that sulphide shuttling is based on sorption to the biomass, and that shuttled sulphide converts to sulphur directly at the electrode surface. Therefore, the sulphide shuttling strategy is not sufficient to prevent electrode passivation, and different strategies need to be applied in the design of a sulphur-producing bioelectrochemical desulphurisation process.

Supporting information may be found in the online version of this article.

Keywords: biodesulphurisation; sulphide oxidising bacteria; bioelectrochemical system; microbial charge storage

INTRODUCTION

Hydrogen sulphide (H_2S) gas is a gas that naturally occurs in biogas, volcanic gas, hot springs and crude petroleum. H_2S itself is toxic and corrosive, causing damage to most living organisms and infrastructure. H_2S can be converted to sulphuric acid and sulphur dioxide upon combustion, causing acid rain and damaging ecosystems.[1,2] H_2S gas is produced in several industries, for example, petroleum, paper and textile industries, but also in agriculture.[3] To prevent sulphide from reaching the environment, technologies have been developed to remove sulphide from waste streams.

Conventional physicochemical sulphide removal strategies involve extreme conditions and/or adding toxic oxidising or precipitation agents and are only economically feasible at high sulphide loading rates.[4] An alternative strategy for sulphide removal is biological desulphurisation, which makes use of the natural ability of sulphide oxidising bacteria (SOB) to oxidise sulphide. For example, sulphide produced by anaerobic digestion of municipal wastewater can be treated by operating the digester at micro-aerobic conditions so the SOB present in the sludge can convert the sulphide to sulphur or sulphate.[5] Gas containing H_2S can be run through biofilters or bioscrubbers, where biofilms containing SOB oxidise the sulphide.[6,7] A few decades ago, biodesulphurisation technology was developed based on absorption of hydrogen gas by a highly alkaline solution and conversion of sulphide to elemental sulphur by SOB under micro-aerobic conditions. This strategy is already globally used in industry.[8-10]

Sulphide can also be oxidised in an electrochemical system.[11-13] Full sulphide oxidation generally proceeds in two steps: sulphide is oxidised to sulphur, releasing two electrons, after which sulphur is further oxidised to sulphate, releasing another six electrons (Table 1). Often, sulphur is the desired product, as it can easily be recovered by settling or centrifugation and can be used as fertiliser in agriculture.[14] Bioelectrochemical removal of sulphide at a bioanode has also been studied.[15,16] Here, microorganisms oxidise sulphide to sulphur while using the anode as an electron acceptor. In these studies, sulphide was available dissolved in solution. When sulphide gets into contact with an anode at a suitable electrode potential, it can be electrochemically converted to sulphur, which is deposited on the anode surface. In time, this sulphur deposition leads to electrode passivation,[11] which is undesirable in practical applications.

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Table 1. Electrochemical sulphide oxidising half-reactions written as oxidation reactions and redox potential at 0.2 mmol L^{-1} sulphide, 0.02 mmol L^{-1} sulphate, pH 8.5 and 30 °C (supporting information F)

Half-reaction	*E* (V *versus* Ag/AgCl)
$HS^- \rightarrow S^0 + 2e^- + H^+$	−0.434
$S^0 + 4H_2O \rightarrow SO_4^{2-} + 6e^- + 8H^+$	−0.590

Recently, it was discovered that mixed SOB from a biological desulphurisation plant can fully remove sulphide when exposed to sulphide under anaerobic conditions.[17,18] Consequently, these SOB that removed sulphide from solution, i.e. sulphidic SOB, released electrons at an anode in an electrochemical cell and thereby acted as 'sulphide shuttles'. The exact mechanisms underlying the spatial separation of sulphide removal and release of electrons are yet unknown; however, sulphide removal is driven by both biological and chemical processes[19] and is reported to reach a maximum of 4–8 mg sulphide per mgN biomass (mgS mgN^{-1}).[18,19] It is hypothesised that the mechanisms behind biotic sulphide removal are based on anaerobic sulphide oxidation and subsequent storage of electrons in electron carriers,[17] and polysulphide formation and subsequent storage in sulphur globules.[19]

Potentially, a new desulphurisation strategy could be designed that uses the sulphide shuttling capacity of SOB (Fig. 1): in the sulphidic compartment, sulphide is dosed and subsequently removed by the SOB, after which the sulphidic SOB-containing charge is transferred to the bioelectrochemical system (BES) resulting in electric current.

Earlier research has investigated the potential of bioelectrochemical sulphide removal using sulphide shuttling SOB. In a batch BES, it was possible to recover charge from sulphide shuttling SOB at a potential of −0.1 V *versus* Ag/AgCl and higher, with an increase in current at increasing potential. Maximal 46 mC mgN^{-1} was recovered from sulphidic SOB, and experiments performed over a long time period of 24 h resulted in a low coulombic efficiency of 13–35%.[17] Laboratory-scale studies of a continuous biodesulphurisation BES with mixed SOB cultures have shown that continuous electron release was possible at a potential of −0.4 to −0.1 V *versus* Ag/AgCl without sulphide present in the anolyte.[20] Biotic runs showed that current production by sulphidic SOB happened at a higher rate and at lower anode potentials compared to abiotic runs. De Rink *et al.*[20] also showed that by replacing oxygen with an anode, the formation of thiosulphate could be prevented. However, selectivity for sulphur production was low (22–44%), and it was postulated that the biofilm that grew on the anode further oxidised sulphur into sulphate.

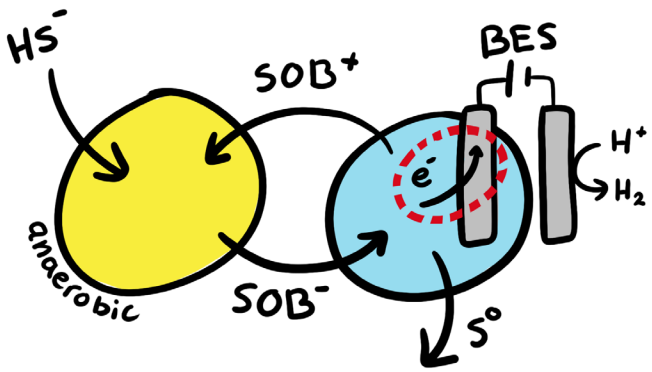

Figure 1. Scheme of the sulphide shuttling strategy. SOB are cycled between the sulphidic reactor (yellow), where sulphide is fed and removed, and the sulphide-free BES (blue) where charge is recovered and sulphur is recovered. The focus of this study, the transfer of electrons to the anode, is circled in red.

This shows that, while integrating a BES into the biodesulphurisation process shows promise, the process needs to be optimised to increase selectivity for sulphur production while maintaining a high sulphide removal rate. To be able to further optimise the bioelectrochemical desulphurisation design focused on sulphur production, it is crucial to have more insights in which SOB have sulphide shuttling capacity or electrochemical activity, in which form sulphide is stored by sulphide shuttling SOB, and what is the contribution of abiotic sulphide oxidation to current production.

Therefore, in the study reported here, the sulphide shuttling mechanism, in particular terminal electron transfer, was investigated by comparing discharge of sulphide shuttling bacteria to abiotic oxidation of sulphide. The interaction with the electrode was studied in an electrochemical system by varying sulphide load, anode potential and, for biotic experiments, biomass concentration. Additionally, different sulphide shuttling SOB communities were compared. Based on the differences and similarities between biotic and abiotic experiments, the sulphide shuttling mechanism of SOB was further elucidated and its implications for future practical implementation are discussed.

MATERIALS AND METHODS

Biomass harvesting

The biomass was harvested from effluent collected from the desulphurisation pilot stationed at Wageningen University (supporting information A). The pilot effluent, containing among others microbial communities, sulphur particles and reduced sulphur species, was collected over a period of multiple weeks at four different time periods: community 1 in December 2020, 2 in April 2021, 3 in February 2022 and 4 in April 2022.

Pilot effluent was centrifuged for 30 min at 7500 × *g* (high-speed centrifuge Z 36 HK, Hermle LaborTechnik, Germany). This resulted in a double-layered pellet. The supernatant was decanted and the red-brown biomass layer was separated from the sulphur layer by carefully resuspending in bicarbonate buffer (1 mol L^{-1} or 84 g L^{-1} $NaHCO_3$ (EMSURE Merck, Germany), pH 8–8.5). The biomass was then washed by centrifuging at 15 000 × *g* and resuspending the biomass again in buffer. The washing step was repeated at least twice or until no sulphur was visible in the pellet. After the last centrifuging step the biomass was resuspended in bicarbonate buffer to make a biomass stock at high concentration (>200 mgN L^{-1}). The biomass stock was sparged with air overnight to remove residual sulphur and to fully oxidise the SOB. The exact biomass concentration was measured using a Hach Lange kit (LCK 138, Hach Lange, USA) and expressed as mgN L^{-1}. A sample was taken and stored at −80 °C for next-generation sequencing. The biomass stock was then stored at 4 °C until use. Experiments were performed within 10 days of harvest.

Next-generation sequencing

DNA extraction was done using a DNeazy power soil pro kit (Qiagen, Germany). The final DNA concentration was checked (Qubit 1x dsDNA HS Assay Kit and Qubit 4 fluormeter, ThermoFisher Scientific, USA). DNA samples were stored at −80 °C until next-generation sequencing was performed. Further amplification of DNA using the V3-V4 of 16s rDNA, as well as DNA analysis

was performed as described previously,[21] using Greengenes 13_8 reference database to pick OTUs and classify the sequences.

Sulphide stock

The sulphide stock was made by dissolving $NaSH{\cdot}H_2O$ flakes (Acros Organics, Belgium) in demineralised water, previously made anaerobic by sparging with nitrogen gas. The sulphide content of the stock was checked by ZnAc redox titration (Titrino Plus, Metrohm, Switzerland) in triplicate, using 0.1 mol L^{-1} $AgNO_3$ solution to titrate 0.1 mL of sample in a mixture of 80 mL of 5% NaOH and 10 mL of 2.5% NH_3.

Sulphide removal and current production

Chronoamperometry

The experiments were performed in a cylindrical 50 mL glass BES with a magnetic stirrer (supporting information B). The anode was a 20 mm × 100 mm graphite felt held in place by a 50 mm × 3 mm diameter graphite rod and connected to an Ag/AgCl reference electrode (QM710X, ProSense, Netherlands) with a capillary and 3 mol L^{-1} KCl salt bridge. The cathode was a 9 mm × 5 mm platinum foil. The electrodes were connected to a potentiostat (Ivium-n-stat, Ivium Technologies, Netherlands). In the BES, the bicarbonate buffer solution with SOB was flushed with nitrogen gas for 15 min to create an anaerobic environment (detection limit of 5 ppb; DP-PSt3, ProSense, Netherlands), after which the sulphide was added with a syringe. The total volume of the biomass–sulphide–buffer solution was 50 mL. All experiments were performed at room temperature (25–28 °C). The experimental conditions are summarised in Table 2. All electrode potentials are given as volts *versus* Ag/AgCl.

To determine the presence of different current regimes, sulphidic SOB were discharged for a longer time period. SOB were made sulphidic by incubating with 0.2 mmol L^{-1} sulphide for 15 min. Thirty mgN L^{-1} sulphidic SOB (microbial community 4) and abiotic run with 0.2 mmol L^{-1} sulphide were done for >20 h (overnight) or until no current was measured, in duplicate. The recovered charge was used to determine the maximum coulombic efficiency (CE). Even though sulphate formation was probable, it was assumed that all sulphide was converted to sulphur to compare the discharge efficiency to earlier research. CE was calculated using the following equation:

$$CE = \frac{\int_0^t I\,dt}{[HS^-]_0 VnF} \times 100\% \quad (1)$$

Here, I is the current (A), t the time (s), $[HS^-]_0$ the sulphide load (mol L^{-1}), V the reactor liquid volume (L), n the number of electrons transferred (2 for sulphur) and F the Faraday constant (96 485 C mol^{-1}). A separate run was done with 30 mgN L^{-1} aerated SOB, 30 mgN L^{-1} sulphidic SOB with 0.2 mmol L^{-1} sulphide and 64 mgN L^{-1} sulphidic SOB with 0.07 mmol L^{-1} sulphide during which liquid samples were taken periodically to measure sulphate and thiosulphate concentration.

To study the effect of anode potential on electrochemical discharge, discharge was measured at a sulphide load of 0.1 mmol L^{-1} and SOB concentration of 30 mgN L^{-1} (community 2). In the reactor, the SOB solution was flushed with N_2 to produce anaerobic conditions. SOB were made sulphidic by incubation with sulphide, during which SOB remove sulphide from the solution. After 20 min a sample was taken to verify the absence of sulphide with lead(II) acetate paper (with a detection limit of 5 ppm). When no coloration of lead(II) acetate occurred, sulphide was not present in the solution and the anode was set at −0.4, −0.1, 0.1, 0.25 or 0.4 V. Current was measured for 10 min, independent of whether steady state was reached. The first 10 s were excluded to exclude most of the capacitive current. Both SOB without sulphide treatment, further called aerated SOB, and sulphidic SOB were measured in duplicate; an abiotic control at 0.1 mmol L^{-1} sulphide was measured in quadruplicate. Due to technical problems, only the first 90 s were logged during the discharge of sulphidic SOB at −0.4 V. To include this measurement, it was chosen to plot the charge collected within the first minute.

To investigate the effect of sulphide load on discharge of sulphidic SOB, discharge was measured for sulphide loads of 0.1, 0.2, 0.5, 1 or 2 mmol L^{-1} at a SOB concentration of 11 mgN L^{-1} (community 1). Fifteen minutes after sulphide was added the solution was sampled to determine the sulphide concentration, as SOB removed sulphide from the solution. The anode was set at 0.1 V. Current was measured for half an hour. Abiotic runs were done using the same sulphide concentration as measured after sulphide removal by SOB to analyse charge that could be recovered from abiotic sulphide oxidation. Experiments were done in triplicate.

To investigate the influence of biomass concentration, SOB were incubated with 0.4 mmol L^{-1} sulphide for 15 min and then discharged at a potential of 0.1 V for 30 min. Biomass concentrations of 0, 30 and 100 mgN L^{-1} (community 3) were used. Measurements were done in triplicate.

Cyclic voltammetry

Cyclic voltammetry was performed on aerated and sulphidic SOB (microbial community 4). A graphite rod with a submerged surface of 617 mm^2 was used as anode, which was rinsed and polished between each run. Anaerobic conditions were reached by sparging the buffer with N_2 gas for 15 min before adding sulphide. SOB that were previously discharged for >20 h during the current regime experiment were used as aerated SOB. To make SOB sulphidic, SOB were fed anaerobically with 0.1 mmol L^{-1} sulphide for 30 min. Afterwards, no free sulphide was detected. Then cyclic voltammetry was done with a potential

Table 2. Summary of parameters used during the experiments performed in this study

Experiment	Potential (V *versus* Ag/AgCl	Duration of chronoamperometry	Sulphide load (mmol L^{-1})	Biomass concentration (mgN L^{-1})	SOB community
Current regimes	0.1	>20 h	0, 0.07, 0.2	0, 30, 64	4
Anode potential	−0.4, −0.1, 0.1, 0.25, 0.4	10 min	0.1	0, 30	2
Sulphide load	0.1	30 min	0.1, 0.2, 0.5, 1.0, 2.0	0, 11	1
Biomass concentration	0.1	30 min	0.4	0, 30, 100	3

step of 1 mV s^{-1} and a potential range of −0.5 to 0.4 V. The cycle was repeated three times and the last cycle was selected for analysis.

Analysis

Samples were filtered with 0.2 µm filter and sulphide was measured using a Hach Lange kit (LCK 653, Hach Lange, USA). The samples were diluted directly in a Hack Lange tube using 0.1 ZnAcetate. The sulphate and thiosulphate concentrations were measured by ion chromatography. The samples were diluted 50 times with ultrapure water (Milli-Q IQ7005, Merck, Germany). The ion chromatography setup consisted of Dionex ICS6000, Autosampler AS-AP, Dionex IonPac AS17-C 2 mm IC analytical column for anion analysis, AS17-C 2 mm guard column and Dionex ADRS 600 2 mm Dynamically Regenerated Suppressor (Thermo Fisher Scientific, USA). The KOH effluent flowed at 0.25 mL min^{-1}, the injection volume was 10 µL and the oven temperature was 30 °C.

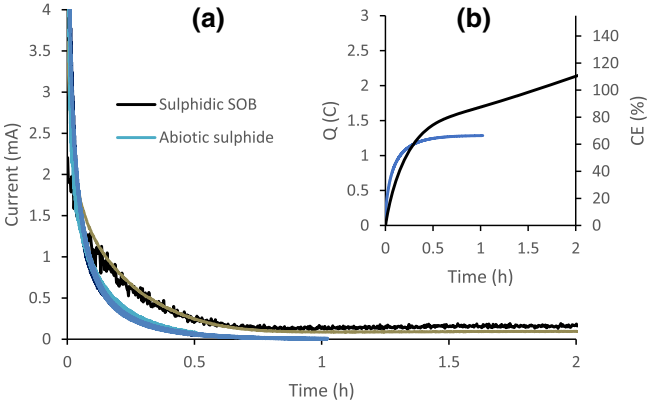

Figure 2. (a) Current of individual replicas and (b) averaged charge recovered and corresponding CE assuming sulphur formation during discharge by 30 mgN L^{-1} sulphidic SOB with 0.2 mmol L^{-1} sulphide (black/grey, $n = 2$, no sulphide was detected in solution) and 0.2 mmol L^{-1} sulphide (shades of blue, $n = 3$) at 0.1 V.

RESULTS AND DISCUSSION

Understanding current production by sulphide shuttling SOB

Current regimes

Batch BES were operated for multiple hours to see the development of current in time and to analyse which part of the charge provided as sulphide could be recovered as electricity. Abiotic sulphide and sulphidic SOB were tested at a sulphide load of 0.2 mmol L^{-1}. After SOB was incubated with sulphide for 15 min, no sulphide was detected in solution, so all sulphide was removed from solution by SOB.

Abiotic sulphide oxidation started at 4 mA and showed an exponential decrease in current in time. After 0.75 h current production decreased to zero and a CE of 65–70% was reached. Possibly, oxidation of sulphide by a low influx of oxygen could account for the remainder of charge.

The current recovered during prolonged discharge of sulphidic SOB showed two distinct current regimes (Fig. 2). During the first 0.75 h the initial current of 2.5 mA decreased exponentially. Then, after 0.75 h a stable current of 0.1–0.2 mA was reached. It is postulated that these different current regimes are dominated by different oxidation processes.

The exponential decline in current during the first 0.75 h observed for sulphidic SOB was not observed for aerated SOB (supporting information D2) and therefore caused by the addition of sulphide. As abiotic sulphide oxidation showed a similar exponential decline in current it is thought that current produced during the first 0.75 h by sulphidic SOB is caused by the same process, i.e. sulphide oxidation. The similarities between current production by abiotic sulphide and sulphidic SOB are further discussed in a later section. Sulphidic SOB are known to produce both sulphur and sulphate when oxidising sulphide.[20] At 0.75 h a CE of 80–85% was reached, assuming all sulphide was converted to sulphur. Compared to similar experiments performed by Ter Heijne *et al.*,[17] where a CE of 13–35% was reached in 24 h, a significant increase in CE was reached by increasing the anode surface area. This is further discussed in the following section.

The stable current observed after 0.75 h discharge of sulphidic SOB of 0.1–0.2 mA was similar to the stable current produced by aerated SOB (supporting information D). Both aerated and sulphidic SOB produced sulphate during discharge (supporting information C) so it is thought that SOB contained sulphur, even after thorough aeration. Therefore it is postulated that the stable current of about 0.2 mA observed for aerated and sulphidic SOB is caused by oxidation of sulphur to sulphate. The formation of sulphate explains why discharge of sulphidic SOB reached a CE of higher than 100% after about 1.5 h.

Anode potential

To investigate the relation between anode potential and current production, sulphide shuttling SOB were exposed to different potentials. Here, 30 mgN L^{-1} sulphidic SOB were prepared by incubating SOB with 0.1 mmol L^{-1} sulphide for 20 min. Afterwards, no sulphide was detected in solution and all sulphide had been removed by the SOB. Exposing the sulphidic SOB to an anode resulted in an electric current. Electric current was collected. The results of charge harvested during the first minute as a function of anode potential are shown in Fig. 3; the individual measurements of current as a function of time can be seen in supporting information D.

Charge recovery during 1 min discharge of sulphidic SOB and abiotic sulphide showed a similar response to potential. At a potential of −0.4 V charge was drawn from the electrode, resulting in negative charge recovery of −25 to −50 mC. However at potentials of −0.1 V and higher the initial current (followed by exponential decay) became positive and increased to 0.2–5 mA with increasing potential, resulting in an increase in charge recovered as potential increased, reaching its highest charge recovery of approximately 200 mC at 0.4 V. Aerated SOB showed a stable low charge recovery of the order of magnitude of 10 mC, which was negative at −0.1 V and positive at higher potentials.

Compared to previous work, the recovery of charge from sulphidic SOB was improved. Ter Heijne *et al.*[17] recovered 68 ± 12 mC of charge during 10 min of discharge at 0.1 V after incubating 30 mgN L^{-1} SOB with 0.2 mmol L^{-1} sulphide. The charge at similar conditions recovered in this study was five times higher (361 ± 47 mC), even though the sulphide load and biomass concentration were halved. The increase in current resulted in a higher CE compared to previous work. At 0.1 V a CE of 33% was reached after 10 min in this work, assuming all sulphide was converted to sulphur. After 10 min, the current had not yet decreased to zero, so the SOB were not yet completely discharged. In comparison, Ter Heijne *et al.*[17] only reached a CE of 13–32% after 24 h of discharge; after this 24 h, current was stable and close to

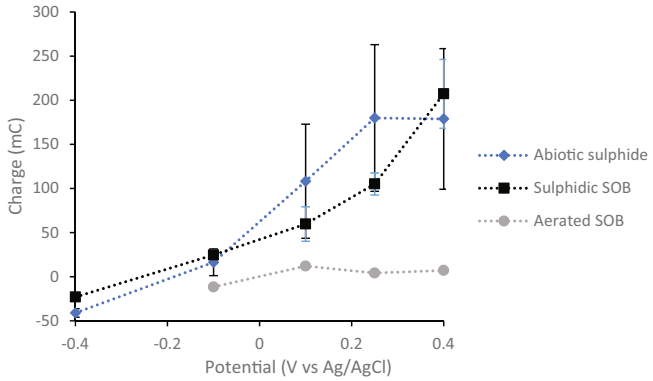

Figure 3. Charge recovered after 1 min of discharge at different potentials of 30 mgN L^{-1} of aerated SOB (grey circles, $n = 2$), sulphidic SOB with 0.1 mmol L^{-1} sulphide (black squares, $n = 2$) and abiotic 0.1 mmol L^{-1} sulphide (blue diamonds, $n = 4$). The dashed lines connect the average to guide the eye. Error bars show standard deviation.

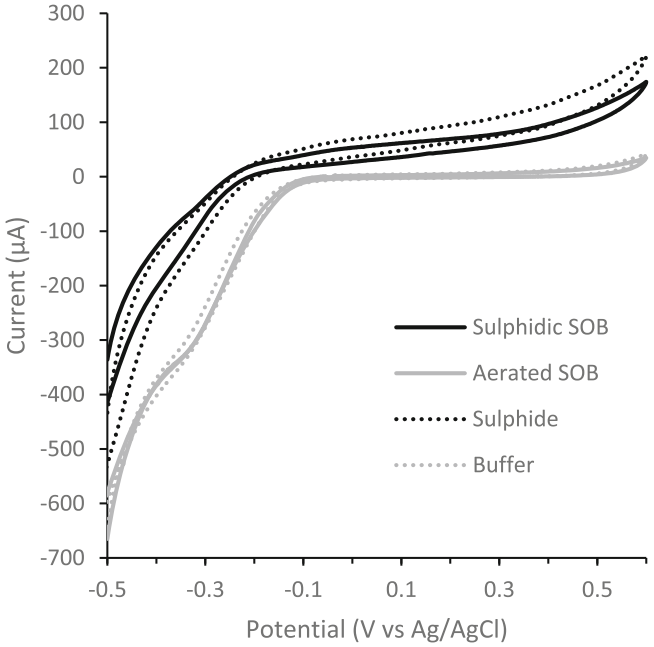

Figure 4. Cyclic voltammetry between −0.5 and 0.6 V *versus* Ag/AgCl at 1 mV s^{-1} of 30 mgN L^{-1} sulphidic SOB with 0.1 mmol L^{-1} sulphide (black line, no sulphide was measured in solution), aerated SOB (grey line), 0.1 mmol L^{-1} sulphide (black dots) and buffer (grey dots). The solution was not stirred during the experiment. Cyclic voltammetry was performed three times. Only the last cycle is shown.

zero and it could be assumed the SOB were fully discharged. Additionally, in this work, more charge was recovered relative to the amount of biomass. At 0.4 V, a total charge of 885 ± 52 mC was recovered in 600 s. This corresponds to a total charge released by the SOB of 600 mC mgN^{-1}, which is 10 times higher than the maximum charge reported by Ter Heijne *et al.*[17]

The reason for the higher current and efficiency is most likely related to anode surface area. The anode used by Ter Heijne *et al.* was a cylindrical rod with a submerged external surface of 3.1 cm^2, while this study uses a graphite felt with a projected surface of 40 cm^2. This converts to 22 mC cm^{-2} charge recovered per projected area in 600 s for both the rod and graphite felt, and thus the current normalised to anode surface area was the same for both anodes. Likely, a further increase in anode surface area would result in a further increase in current.

To further investigate the interaction between SOB, sulphide and the anode, we performed cyclic voltammetry on sulphidic and aerated SOB, abiotic sulphide and buffer (Fig. 4).

The buffer showed negligible current at potentials higher than −0.1 V. Below −0.1 V, the current became more negative as the potential went down. Reduction of bicarbonate buffer was not expected, as bicarbonate is reduced at potentials lower than applied in this study.[22] It is not known which compounds were reduced at potentials lower than −0.1 V. To test whether the products formed during reduction influenced current production at higher potentials, the cyclic voltammetry was performed in a potential window of −0.15 to 0.6 V (supporting information E). No effect on oxidation current was observed.

The voltammogram for aerated SOB was very similar to the voltammogram for the buffer alone which showed that aerated SOB did not interact with the electrode at these conditions. In the presence of sulphide, the current became positive at potentials higher than −0.2 V, which is in line with the batch results, and reached currents of 100–200 µA. The cyclic voltammetry curves of sulphide do not show any clear peak that can be attributed to sulphide oxidation. Possibly, the influx of a low amount of oxygen could have suppressed subtle features of the voltammogram. Interestingly, the voltammogram of sulphidic SOB, in which all sulphide was removed from solution, was very similar to that of abiotic sulphide: current was produced at potentials of −0.2 V and higher, but no peaks or waves were observed in the cyclic voltammetry. This is surprising since it was expected that discharge by SOB involved either additional reduced sulphur compounds, such as polysulphide, or enzymes and would thus show (additional) peaks. Further discussion on the lack of enzyme peaks and other similarities between electrochemical sulphide oxidation and discharge of sulphide shuttling SOB can be found in a later section.

Thermodynamic calculations (supporting information F) show that at 0.1 mmol L^{-1} sulphide, pH 8.5 and 30 °C, oxidation of sulphide to sulphur is energetically favourable at −0.425 V *versus* Ag/AgCl. As at the applied conditions oxidation current only occurred at potentials around −0.2 V and higher, the overpotential needed for sulphide oxidation as observed was *ca* 0.22 V. It is not known how potential influences product formation during sulphide oxidation; however, thermodynamically sulphate production is more favourable than sulphur formation at potentials relevant for sulphide removal. Therefore, in future design of a bioelectrochemical desulphurisation process, it does not seem feasible to steer sulphide oxidation towards sulphur production by varying the anode potential.

Sulphide load

The effect of different sulphide loads on charge recovered from sulphidic SOB was investigated by applying a potential of 0.1 V for 30 min after incubating SOB with different sulphide loads (Fig. 5). SOB were made sulphidic by incubating with sulphide for 15 min. Only at the lowest sulphide load of 0.1 mmol L^{-1} was all sulphide removed from solution in 15 min. Thus at higher sulphide loads, sulphide was still present when potential was applied. In that case, the total measured charge of sulphidic SOB partly came from charge stored within SOB and partly from sulphide in solution. To distinguish between the two, abiotic runs were performed with sulphide concentrations measured after incubation of SOB. The abiotically produced charge (blue in Fig. 5(a)) was then subtracted from

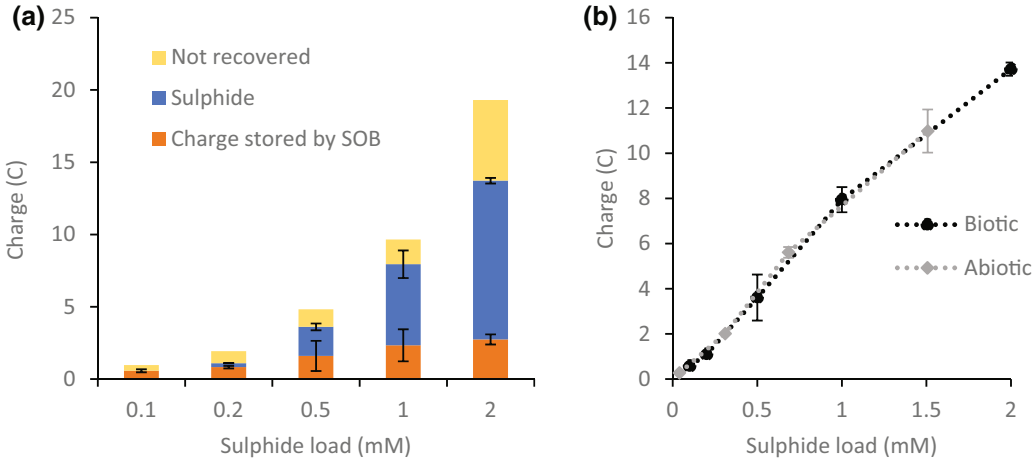

Figure 5. (a) Charge recovered in 30 min of exposure to 0.1 V after incubating 11 mgN L^{-1} SOB for 15 min with different sulphide loads. Charge came from abiotic sulphide oxidation (blue) or release of charge stored by SOB (orange). The total bar shows the maximal charge that could be recovered assuming all sulphide was converted to sulphur. Part of the electron balance could not be closed (yellow). (b) Charge recovered in 30 min at 0.1 V of different sulphide loads in the absence (grey diamonds) or presence (black circles) of SOB. The dotted lines connect the average to guide the eye. The error bars show the standard deviation (n = 3).

total charge produced by sulphidic SOB (the sum of blue and orange) to determine the charge recovered from charge stored by SOB (orange).

The total charge recovered of sulphidic SOB (blue + orange) increased proportional to sulphide load, showing a fairly constant CE of 70 ± 5%. At the end of the experiment, current was not decreased completely to zero, and therefore the remaining 30% non-recovered charge (yellow) could still be present in the form of sulphide or stored charge. The average charge that was recovered from charge stored by SOB (orange) increased with sulphide load but did not increase significantly after 0.5 mmol L^{-1} $[HS^-]_0$. The highest charge recovered from SOB was 2.7 ± 0.35 C, or 4900 ± 810 mC mgN^{-1}.

How charge is stored by SOB is still unknown. However, when plotting the recovered charge *versus* sulphide load for both abiotic and biotic experiments (Fig. 5(b)), both showed a very similar linear dependency on sulphide load. Whether sulphide was stored by SOB or was present in solution did not seem to be of influence for the total charge and current. Thus, no distinction could be made between electron transfer by abiotic sulphide oxidation and electron release by sulphidic SOB. These findings are further discussed in a later section.

Biomass concentration

The effect of biomass concentration on sulphide removal and charge recovery was investigated at 0, 30 and 100 mgN L^{-1} incubated with 0.4 mmol L^{-1} sulphide (Fig. 6). In the presence of biomass, about 25–60% of the sulphide was removed in 15 min. Increasing the biomass concentration from 30 to 100 mgN L^{-1} did not significantly increase sulphide removal. Earlier research showed that sulphide removal is only partly dependent on biological processes. After a certain biomass and/or sulphide concentration, chemical processes and equilibria become limiting.[19] Which chemical processes play a role is not yet known, though it is postulated that the equilibrium between sulphide, sulphur and polysulphides is involved.

The charge recovered during 30 min of discharge at 0.1 V was 1.8–3 C, resulting in a CE of 130–160% when assuming oxidation of sulphide to sulphur. Sulphide oxidation towards sulphate instead of sulphur releases six more electrons; therefore it is likely that aside from sulphur, also sulphate was formed, resulting in CE > 100% (not confirmed). The total charge recovered was not influenced by the increase in biomass concentration. Interestingly, also in these experiments, it was found that the current and total charge produced from abiotic sulphide are similar to the current produced from sulphidic SOB (supporting information G), which is further discussed in a later section.

Biomass composition of sulphide shuttling communities

The experiments as described in the previous section were performed with four biomass stocks harvested from a pilot biodesulphurisation plant stationed at Wageningen University (Table 2). The biomass used for the batch experiments was harvested at different times within a time period of 1.25 years, in which the microbial community of the pilot changed. Using next-generation sequencing, the relative abundance of the species in the microbial communities used in this study was determined (Fig. 7).

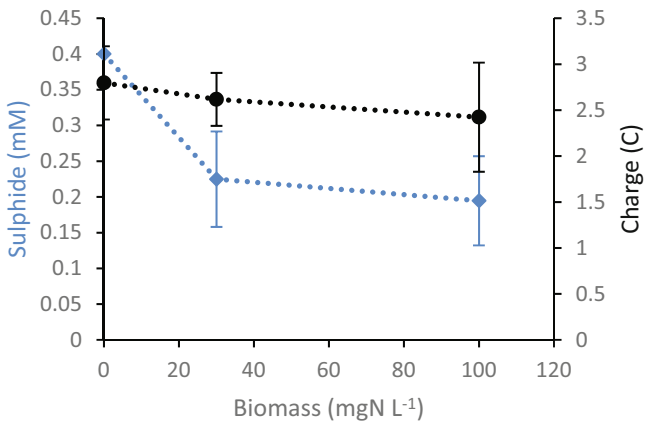

Figure 6. Sulphide in solution (blue diamonds) after 15 min of incubation of 0.4 mmol L^{-1} sulphide with 0, 30 or 100 mgN L^{-1} SOB and charge recovered (black circles) during 30 min of discharge at 0.1 V *versus* Ag/AgCl after incubation. The dashed lines connect the average to guide the eye. Error bars show standard deviation of triplicates.

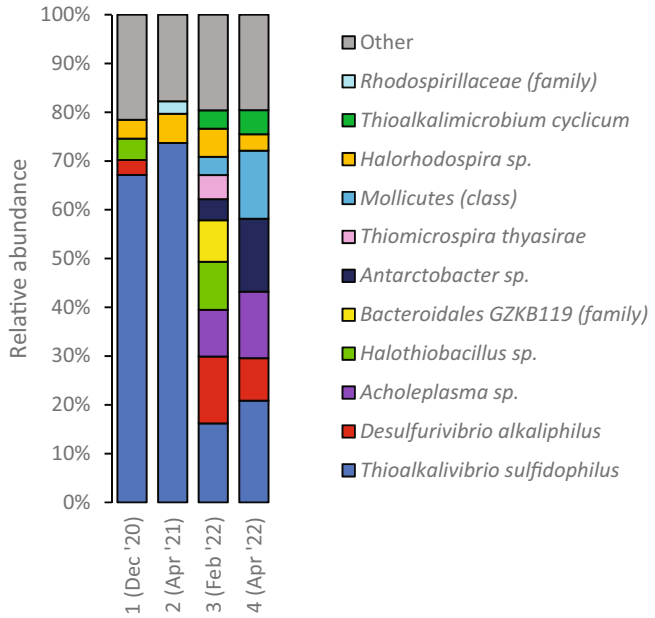

Figure 7. Relative abundance of the microbial communities harvested from a pilot desulphurisation plant at different time points. Only species or families with a relative abundance of at least 2.5% are shown.

We assume that no significant growth took place during the performance of the batch experiments with a duration of up to 24 h.

The dominant species in all communities was *Thioalkalivibrio sulfidophilus*, with a relative abundance of 65–75% in communities 1 and 2, and 16–20% in communities 3 and 4. All communities contained *Halorhodospira* sp. (3–6%). Other species detected were *Desulfurivibrio alkaliphilus* (9–14%), *Acholeplasma* sp. (10–14%), *Antarctobacter* sp. (4–15%), unclassified *Mollicutes* (4–15%) and *Thioalkalimicrobium cyclicum* (4–5%). Also, *Halothiobacillus* sp. (10%), *Bacteroidales GZKB119* (9%) and *Thiomicrospira thyasirae* (5%) were detected in community 3 and species of the family Rhodospirillaceae (3%) were detected in community 2. In previous research on sulphide shuttling SOB, *Alkalilimnicola ehrlichiii* was one of the dominating species, but interestingly this species was not detected in this study.

Most of the species found in the microbial communities are known or suspected to be able to oxidise sulphide, among them being *Thioalkalivibrio sulfidophilus*, *Desulfurivibrio alkaliphilus*, *Halothiobacillus* sp., *Thiomicrospira thyasirae*, *Thioalkalimicrobium cyclicum* and *Halorhodospira* sp.[23-26] Not much is known about the electrochemical activity of isolated species. Only *Desulfurivibrio alkaliphillus* has been shown to be electrochemically active in pure culture.[27] However, many of the occurring species have been detected in electrochemically active biofilms or planktonic cultures, such as *Thioalkalivibrio sulfidophilus*, *Acholeplasma* sp. and *Bacteroidales GZKB119*[15,20,28-30] (Table 3).

During this study, different microbial communities were used. Communities 1 and 2, harvested during the first quarter of 2021, were similar in composition, with the majority of the community composed of *Thioalkalivibrio sulfidophilus*, whereas microbial communities 3 and 4, harvested in the first quarter of 2022, were more diverse. Despite the differences in microbial communities, current production by all sulphide shuttling communities showed similar and consistent results, which is further discussed in the following section.

It is known that sulphide removal is a biological process.[19] As *Thioalkalivibrio sulfidophilus* was the dominant species in all microbial communities used in this study, it is plausible that this species was the main contributor to sulphide removal. In earlier work, sulphide removal by community 2, with a relative abundance of 75% *Thioalkalivibrio sulfidophilus*, has been extensively investigated and reached a sulphide removal capacity of 8 mgS mgN^{-1} at a total biomass concentration of 0.6 mgN L^{-1}.[19] Assuming relative abundance correlates with relative contribution to total N concentration, *Thioalkalivibrio sulphidophilus* was present in concentrations varying from 5 to 25 mgN L^{-1}. Therefore changes in concentrations of *Thioalkalivibrio sulfidophilus* could have resulted in discrepancies in anaerobic sulphide removal. Nevertheless, all communities were able to remove up to 0.35 mmol L^{-1} sulphide, and fully remove up to 0.2 mmol L^{-1} sulphide in anaerobic conditions. To further elucidate the sulphide shuttling abilities of *Thioalkalivibrio sulfidophilus*, different omics technologies might be of use.

Hypothesised mechanisms for electrochemical discharge of SOB

If current production by SOB could be integrated into the biodesulphurisation process, this would bring several benefits. (i) Using the sulphide shuttling ability of SOB, charge can potentially be harvested from sulphide without sulphide coming into contact with the electrode, thereby preventing electrode passivation. (ii) No energy needs to be put into oxygenation and energy can be recovered from sulphide in the form of electricity, making the process more energy efficient. Previous research showed that it was possible to continuously recover charge in a biodesulphurisation BES run with sulphide shuttling SOB without sulphide present in the anolyte.[20] However, the CE and sulphur recovery were low. As sulphur is the desired end-product, it is necessary to improve the bioelectrochemical desulphurisation process. To make this possible, more insight into the sulphide shuttling mechanisms is required.

Therefore, in this work, an attempt was made to further elucidate the sulphide shuttling mechanisms, focusing on terminal electron transport to the electrode, by comparing current response of abiotic sulphide oxidation and discharge of sulphidic SOB to changes in process conditions. Among factors investigated, only electrode potential and sulphide load had an effect on current production, while neither the concentration of SOB nor the composition of the microbial community were of influence. Additionally, abiotic sulphide and sulphidic SOB showed similar responses to cyclic voltammetry and chronoamperometry, even though in some of the cases no sulphide was detected in sulphidic SOB solutions. Based on these findings, two hypotheses were formulated (Fig. 8).

Hypothesis 1: Free sulphide and charged SOB have similar electrochemical properties.

Ter Heijne *et al.*[17] postulated that anaerobic oxidation of sulphide could explain the removal of sulphide in the absence of oxygen or other terminal electron acceptors. Sulphide could be oxidised using electron mediators such as quinones and cytochromes as electron acceptor. When again exposed to oxygen or an electrode, the electron carriers oxidise and are recycled. In this process, sulphide is oxidised to sulphur or sulphate and electrons are stored in electron mediators, and therefore no sulphide is present intra- or extracellularly. Additionally SOB could store sulphide in sulphur globules, sulphur stored intra- or extracellularly in a protein envelope. Sulphide could react irreversibly with the sulphur to form short-chain

Table 3. Species found with a relative abundance of over 2.5% in microbial communities used in this study with corresponding sulphide metabolism and electroactivity according to literature

Species	Sulphide metabolism	Electroactivity
Thioalkalivibrio sulfidophilus	Complete sulphide oxidation[23]	Detected as dominant species in anodic electroactive planktonic biomass[17,20]
Desulfurivibrio alkaliphilus	Sulphate reduction, elemental sulphur disproportionation, oxidation of sulphide to elemental sulphur[26]	Shows bioelectrochemical catalysis in both anodic and cathodic conditions[27]
Acholeplasma sp.	Not reported	Suspected to be the functional electrochemical microorganisms in denitrifying bio-cathodes[28]
Halothiobacillus sp.	Complete sulphide oxidation[23]	Not reported
Antarctobacter sp.	The Rhodobacteraceae family might include photoheterotrophic anaerobic SOB[20]	Some Rhodobacteraceae were isolated from electroactive biofilms[31]
Bacteroidales GZKB119 (family)	Not reported	Bacteroidales have been detected in anodic and cathodic biofilms[29,30]
Thiomicrospira thyasirae	Complete sulphide oxidation[23]	Not reported
Mollicutes (class)	Not reported	Found as dominant species in cathodic conditions[32]
Halorhodospira sp.	Genes present in genome for sulphide oxidation and sulphate reduction[24]	Not reported
Thioalkalimicrobium cyclicum	Complete sulphide oxidation[25]	Not reported
Rhodospirillaceae (family)	Some genera are known to oxidise sulphide and thiosulphate[33]	Detected as dominant family in anodic biofilms[34]

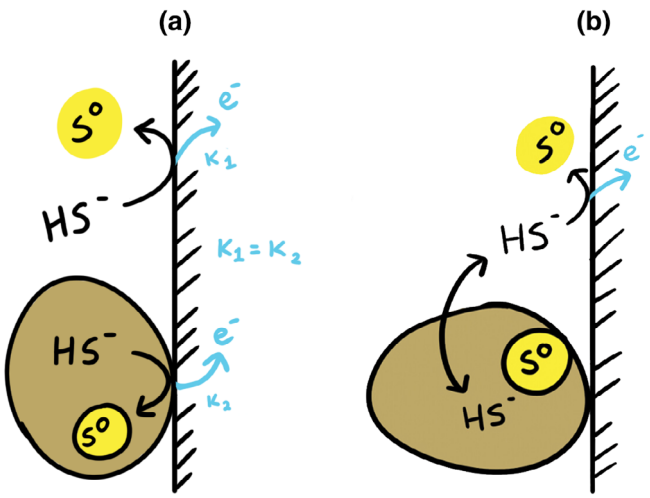

Figure 8. Postulated mechanics of current production during discharge of sulphide shuttling bacteria. (a) Discharge of sulphide removed by SOB and abiotic oxidation of sulphide in solution show similar kinetics, possibly due to reactor limitations. (b) Sulphide removed from solution by SOB is again released when SOB approach the anode, after which sulphide is oxidised abiotically.

polysulphides, which accumulate in the sulphur globules. Upon introduction with oxygen or an electrode these polysulphides are again oxidised.[19]

In both cases, oxidation could proceed via biological pathways using a combination of electron mediators and membrane-bound redox active proteins, mainly c-type cytochromes, to facilitate direct extracellular electron transfer (DEET).[35] The first hypothesis is thus that DEET or direct bioelectrochemical sulphide oxidation are similar to electrochemical oxidation of sulphide in terms of kinetics. This could indicate that the similarities between abiotic and biotic oxidation kinetics in this work were caused by limiting reactor properties. For example, mass transfer, the electrode surface area or conductance could have limited current production. An improvement in bioreactor design could then lift these limitations and allow for observation of the differences between electron transfer kinetics of sulphide and sulphidic SOB.

Additionally, it is of importance to investigate the relation between anaerobic sulphide removal and redox state of the electron mediators in sulphide shuttling SOB. Raman spectroscopy for example is able to measure the redox state of cytochromes in live cells.[36] Raman spectroscopy could also be used to determine the content of sulphur globules.[37]

Nevertheless, at a sulphide loads above 0.2 mmol L^{-1}, a significant fraction of sulphide was not removed from solution and therefore in direct contact with the anode during discharge. To prevent anode passivation the sulphide load should be kept low and/or the removal of sulphide by SOB should be increased to prevent direct contact between sulphide and anode. Increasing SOB concentration could allow for a higher sulphide load while avoiding anode passivation, but after a certain SOB concentration sulphide removal cannot be further increased by increasing SOB content.[19] It is shown that by shuttling SOB between anaerobic sulphidic and aerobic conditions the sulphide removal capacity is increased.[38] Potentially an enrichment routine based on these changing anaerobic and aerobic conditions could be designed to further select for SOB with higher sulphide removal capacities.

Hypothesis 2: Sulphide is stored and again released when cells approach the anode.

The similarities between kinetics of sulphidic SOB and abiotic sulphide lead to a second hypothesis: for *both* abiotic and biotic conditions, the current is produced by abiotic oxidation of sulphide. It is observed that sulphide is removed from solution in the presence of SOB; however, it is not known what exactly happens to the sulphide after sulphide removal. Possibly, the

sulphide attaches to the outside of the SOB, is stored as intracellular sulphide or is stored in sulphur globules in the form of polysulphides, the product of the equilibrium reaction with sulphur. When the SOB approach the anode, the sulphide could be released and/or come into contact with the electrode surface and subsequently oxidised at the anode in the same way as sulphide would in the absence of SOB, without SOB directly playing a role in oxidation (Fig. 8). In this case, the electrochemical activity of the microorganisms present is not relevant for selection of sulphide shuttling bacteria, and although *Thioalkalivibrio sulfidophilus* dominated all sulphide shuttling communities used in this research, its electroactivity cannot be proven.

Storage of sulphide in the absence of electron acceptors by sorption could be a survival strategy. When exposed to intermittent anaerobic sulphidic and aerobic non-sulphidic conditions, sulphide shuttling SOB have an advantage over other, non-sulphide shuttling, SOB: in aerobic non-sulphidic conditions sulphide shuttling SOB can metabolise their sorbed sulphide. However, this survival strategy does not hold in a BES designed on sulphide shuttling (Fig. 1). Here, sulphide is sorbed to the cells and later electrochemically oxidised, and thus the electrons from sulphide are not available for the respiratory chain of SOB. This would be energetically disadvantageous for SOB and would not support growth.

A continuous BES that showed sulphide shuttling behaviour was successfully operated,[20] with sulphate as main product. Further steering product formation to sulphur could result in complications with biomass growth, but also with electron transfer to the anode surface. The sulphur formed at the anode by direct electrochemical oxidation of sulphide may result in electrode passivation. Strategies to prevent sulphur deposition could include changing the design of the anode using appropriate electrode material,[35] surface structure[12] or reactor design.

Whether SOB would release sulphide back into solution is currently unknown, and the formation of sulphur could not be confirmed in this study due to low sulphide concentrations and low conversion rates. To shed more light on this hypothesis and its role in discharge of sulphidic SOB, further experiments need to be performed. A rotating disc electrode provides control over mass transport towards the electrode. Therefore redox behaviour of adsorbed, dissolved and suspended redox species can be investigated. The rotating disc could be used to gain more insight into the current production by sulphidic SOB while showing sulphur precipitation at the electrode surface. Also, scanning electron microscopy combined with energy-dispersive X-ray spectroscopy of SOB could be used to investigate the presence or absence of membrane-bound sulphur. Additionally, the sorption and release of sulphide by SOB could be further investigated with membranes which keep biomass away from the electrode but allow small molecules, i.e. sulphide, to diffuse to the electrode surface.

CONCLUSION

SOB were incubated with sulphide for 15 min, after which sulphide was (partly) removed from solution. Different microbial communities, all dominated by *Thioalkalivibrio sulfidophilus*, were investigated. Despite the differences in microbial communities, anaerobic sulphide removal and oxidation at the anode showed similar and consistent results.

In sulphidic conditions, current was produced at potentials of −0.2 V *versus* Ag/AgCl and higher in the absence and presence of SOB. The CE was independent of sulphide load and reached about 70% in half an hour. Recovery of charge stored by SOB reached a maximum of 4900 ± 810 mC mgN^{-1} at a sulphide load of 0.5 mmol L^{-1}, which is a significant improvement compared to earlier findings.

By comparing current production of sulphidic solutions in the absence and presence of SOB, more knowledge is gained on the discharge kinetics of sulphide shuttling SOB. In sulphidic conditions, the biomass content and degree of sulphide removal did not influence discharge kinetics. Sulphidic SOB and abiotic sulphide showed similar responses to chronoamperometry and cyclic voltammetry, even though in the presence of SOB (part of the) sulphide was not detected in the solution. It is postulated that sulphide is sorbed to the SOB and released as SOB approach the anode, after which sulphide is oxidised at the anode, possibly depositing sulphur on the electrode surface. This would imply that the sulphide shuttling strategy is not sufficient to prevent electrode passivation, and different strategies need to be applied in the design of a sulphur-producing bioelectrochemical process. More research is needed to provide proof for the proposed sulphide shuttling mechanism and its associated deposition of sulphur on the electrode, which form the basis of assessment of the feasibility of bioelectrochemical desulphurisation.

AUTHOR CONTRIBUTIONS

Rikke Linssen: Methodology, Investigation, Formal analysis, Visualisation, Writing – original draft. Annemiek ter Heijne: Supervision, Funding acquisition, Writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

Data used in this study can be found in DOI: 10.4121/f6bd7e7c-ffa8-4590-96db-ca870a2c0847.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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