# Deciphering the fate of sulfate in one- and two-chamber bioelectrochemical systems

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## Abstract

Treatment of wastewaters with high concentration of sulfate before discharge is an environmental imperative. However, current technologies are often highly energy and chemical-demanding. Recently, microbial electrochemical technologies (MET) have been proposed as an alternative and more sustainable approach for the treatment of wastewaters rich in sulfate. Here we compare the sulfate reduction performance of one- and two-chamber reactors. In two-chamber reactors, sulfate reduction to sulfide was achieved with high electron recovery efficiency (83.9 ±1.3 %) at a sulfate reduction rate of 9.7 ±2.6 $mgSO_4^{2-}$ $L^{-1}$ $d^{-1}$ $cm^{-2}$, whereas in one-chamber reactors apparently no sulfate reduction took place. Non-electrochemical microcosm experiments suggested that sulfate reduction in two-chamber reactors was driven by the availability of cathodically produced $H_2$. In one-chamber reactors, the presence of anodically produced $O_2$ presumably resulted in chemical short circuit including aerobic hydrogen oxidation, abiotic and biotic re-oxidation of sulfide to sulfate. Microbial community analysis demonstrated that sulfate reduction was mainly performed by sulfate reducing prokaryotes (SRP) belonging to the genera *Desulfomicrobium* and *Desulfovibrio*, whereas sulfide oxidizing bacteria (SOB) (mainly *Acinetobacter* and *Sulfuricurvum*) could be responsible for re-oxidation of sulfide. Thus, fundamental and application-oriented

research on microbial electrochemical sulfate reduction ought to be studied in two-chamber reactors for avoiding a potential bias of results by anodic reactions.

**Key words**: bioelectrochemical systems, microbial electrochemical technologies, hydrogen-mediated electron uptake, mixotrophic sulfate reduction, sulfur cycle

## 1. Introduction

Wastewaters with high concentrations of sulfate ($SO_4^{2-}$) are produced by a plethora of industries, such as production of pulp and paper, mining, food, and detergents [1]. Its untreated discharge causes environmental pollution and threats human health [1]. Thus, treating sulfate-rich wastewaters prior to discharge is mandatory. Prevailing physiochemical treatment technologies include reverse osmosis, processes based on ettringite formation, and precipitation with barium that are commonly characterized by high demand of energy, chemicals and hence operational expenditures (opex) [2]. An alternative to physicochemical treatment is biological sulfate removal that may offer the advantage of high efficiency at low capital expenditures (capex) and opex. For the treatment of sulfate-rich wastewater under anoxic conditions, the metabolism of sulfate-reducing prokaryotes (SRP) is exploited. In fact, SRP use sulfate as terminal electron acceptor leading to the formation of bisulfide ($HS^-$) and sulfide ($S^-$) that are most often both summarized under the terminus sulfide within this article (Eq. 1) at pH-neutral conditions [3]. Heterotrophic SRP use organic compounds as both, electron donor and carbon source, while autotrophic SRP use $H_2$ as electron donor and $CO_2$ as the carbon source. Notably, many hydrogenotrophic SRP need acetate in addition to $CO_2$ for growth [4]. Typically, sulfate-rich wastewaters are limited in the availability of electron donors, hence creating the need to add carbon sources and electron donors

like $H_2$ or lactate [2] to drive the complete reduction of sulfate, which in turn may considerably increase the opex [5].

$$SO_4^{2-} + 8e^- + 9H^+ \rightarrow HS^- + 4H_2O \quad \text{(Eq. 1)}$$

Consequently, using electric energy as sustainable means at low opex for treating sulfate-rich wastewater has come into play. Abiotic electrochemical sulfate reduction seems no valid technical option, as temperatures higher than 100°C are needed [6]. In contrast, microbial electrochemical technologies (MET) that combine microbial and electrochemical conversions [7, 8] represent a promising alternative for treating sulfate-rich wastewaters. In primary MET, electroactive microorganisms (EAM) [9] that have their metabolism wired to electrodes via extracellular electron transfer (EET) are exploited. The reactors are termed bioelectrochemical systems (BES). Potentiostatically controlled BES are also denominated as microbial electrolysis cells (MEC) and are usually used for harvesting $H_2$ (via the hydrogen evolution reaction (HER) at the cathode) and conducting fundamental research [10]. Primary MET may also provide a solution for the treatment of sulfate-rich wastewaters by microbial electrochemical sulfate reduction [11]. However, the main share of former studies thereon investigated sulfate reduction being accompanied by oxidation of organics by heterotrophic SRP [12-14]. Yet, alternatively using $H_2$ as electron donor for autotrophic SRP seems more favorable as the HER can proceed using biotic [15] or abiotic [16] cathodes in MEC. The ability of autotrophic SRP of scavenging $H_2$ for sulfate reduction has been shown [17]. Noteworthy, the reduction of sulfate as an electron acceptor at microbial cathodes without electron donor was reported by Su and colleagues [18]. However, sulfate removal in BES leads to high concentrations of sulfide at cathodes which is toxic to microorganisms [19-21] and may reduce the overall performance [22]. Biological sulfide oxidation will decrease the sulfide concentration, hence preventing toxicity problems. Recently, the cathodic sulfate reduction

coupled with anodic sulfide oxidation to recover elemental sulfur ($S^0$) was reported. Blazquez and colleagues studied an autotrophic biocathode for reduction of sulfate to sulfide and recovered elemental sulfur from aerobic oxidation of sulfide. The oxygen diffused from the anode chamber to cathode chamber resulting in oxidation of sulfide and partial $S^0$ recovery [20, 23].

Table 1 summarizes the experimental designs of previous studies (pH, cathode potentials, inoculum, cathode material, and reactor configuration) for studying the microbial electrochemical sulfate reduction and illustrates the diversity thereof. One main aspect that can be believed to shape, if not dominate, the sulfur-related redox processes is the use of one- or two-chamber reactors. Whereas in two-chamber reactors a membrane separates the cathode (hosting microbial electrochemical sulfate reduction) and anode compartment, this is not the case in one-chamber reactors. This is of special importance, as the anode catalyzes the oxygen evolution reaction (OER) from water oxidation representing a suitable electron acceptor sulfide oxidation [24].

83 Table 1. Summary of operational parameters of sulfate reduction in microbial electrochemical systems.

| Number of chambers | Cathode Potential (V vs. SHE) | pH | SRR[1] (mg $L^{-1}$ $d^{-1}$) | Inoculum | Dominant organisms | Additional electron donor | Electron recovery[2] (%) | Cathode material | Refer-ence |
|---|---|---|---|---|---|---|---|---|---|
| 2 | -0.9 | 3-7 | 57 | Sediment from Pearl River | *Clostridium* sp. *Desulfovibrio* sp. | Acetate in anode chamber | 25 ±1.8 | Graphite brush | [25] |
| 2 | -0.8 | 6.0 | 152 | Acclimated sediment | *Desulfovibrio* sp. | $H_2$ | 56.1 ±4.1 | Graphite plate | [22] |
| 2 | -0.26 | 10 | 20 | Effluent from MFC treating acetate-enriched wastewater | - | $H_2$ | - | Graphite granules | [26] |
| 2 | - | 3-6.6 | 12.3 | Sediment from Peal River | dominated by *Desulfovibrio* sp. | Acetate in anode chamber | - | Graphite brush | [27] |
| 2 | -0.8 | 6.3-8.3 | 73-112 | Biomass from a sewer system | *Desulfovibrio* sp. *Sulfuricurvum* sp. | No | - | Graphite brush | [20] |
| 1 | -0.61 to -0.81 | 6-7.5 | 12-42 | *Desulfovibrio caledoniensis* | - | Lactate | - | Steel | [28] |
| 2 | -0.36 to -0.76 | 7 | 0.33-8.58 | Domestic wastewater | - | Acetate in anode chamber | 5.3-50 | Graphite brush | [29] |
| 2 | 0-3.5 mA | 7 | max. 140 | Sewage sludge | *Desulfovibrio* sp. | Ethanol | - | Graphite rod | [30] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.4 | 2.5-10.5 | 1.4-14.9 | Acclimatized sludge | *Paludibacter* sp.<br>*Desulfovibrio* sp. | Ethanol | - | Activated carbon cloth | [31] |
| 2 | - | 6.9 | 219 | Activated sludge | - | Lactate | - | Carbon cloth | [14] |
| 2 | -0.35 to -0.47 | 7 | max 7.6 | Sewage sludge | *Acinetobacter* sp.<br>*Desulfovibrio* sp. | Ethanol | 54.2 | Graphite rod and graphite felt | [32] |
| 1 | -0.8 | 7 | - | Sediment from a pond | *Desulfomicrobium* sp.<br>*Desulfovibrio* sp.<br>*Acinetobacter* sp. | No | - | Titanium covered by platinum | This study |
| 2 | | | 97 ± 26 | | *Sulfuricurvum* sp. | | 83.9 ±1.3 | | |

[1]SRR represents sulfate reduction rate.

[2]Electron recovery efficiency is also described as cathodic coulombic efficiency.

Surprisingly, so far the role of the OER on the cathodic sulfate reduction has not been systematically assessed. Thus, the objective of this study was to investigate comparatively the performance of one- and two-chamber reactors in terms of sulfate reduction and sulfide formation. Furthermore, anoxic non-electrochemical microcosm experiments were performed with microbial communities derived from BES to elucidate the role of anodically produced $O_2$ and cathodically produced $H_2$ upon biotic and abiotic reactions of inorganic sulfur compounds. Subsequently, microbial structure-function relationships within the different reactor configurations were analyzed by 16S rRNA gene amplicon sequencing.

## 2. Materials and methods

### 2.1. General remarks

All chemicals were of analytical grade and purchased from Sigma-Aldrich (United States of America), Merck (Germany), and Carl Roth (Germany). If not stated otherwise, all provided potentials refer to the standard hydrogen electrode (SHE) by conversion from Ag/AgCl sat. KCl reference electrodes (+0.197 V vs. SHE).

### 2.2. Medium composition and source of inoculum

Anoxic mineral salt medium (MSM) buffered with $CO_2$/$NaHCO_3$ (30 mM) at pH 7 was used for all experiments containing 0.5 g $L^{-1}$ NaCl, 0.5 g $L^{-1}$ $KH_2PO_4$, 0.4 g $L^{-1}$ $NH_4Cl$, 0.4 g $L^{-1}$ KCl, 0.1 g $L^{-1}$ $CaCl_2$, 0.5 g $L^{-1}$ $MgCl_2$, 5 mL vitamin solution, and 3 mL trace element solution (details in SI 1.2). If required, 10 mM sodium sulfate was added. MSM was prepared using deionized water, and autoclaved at 121°C for 20 min (HV-25, HMC Europe GmbH, Germany). Most MSM compounds dissolved in water before it was flushed by $N_2$ for 120 min to ensure anoxic conditions.

In an anaerobic glove box (95 % $N_2$/5 % $H_2$ atmosphere, Coy Laboratory Products, U.S.A.) 30 mL of a $CO_2$-saturated 1 M $NaHCO_3$ solution and 3 mL trace element solution were added.

The sulfate-reducing inoculum used in experiments with BES and in the sulfide-oxidizing microcosm experiments were taken from enrichment cultures developed and maintained as described in SI 1.1. Prior to the transfer into BES or microcosm experiments, the enrichment culture was anaerobically centrifuged (10,000 g) at 4°C and resuspended in MSM.

2.3. Design and operation of bioelectrochemical systems (BES)

All microbial electrochemical experiments were carried out in BES consisting of four-neck round-bottom flasks (Lenz Laborglas GmbH & CO.KG, Germany) with a working volume of 250 mL either as one- or two-chamber reactor that is with or without a membrane. Both cathode (working electrode, WE) and anode (counter electrode, CE) were Pt-covered titanium electrodes (PLATINODE®, Umicore Electroplating, Germany) soldered to a titanium wire (Goodfellow GmbH, Germany) covered by a polytetrafluoroethylene tube (Shrink-Kon®, Thomas & Betts Corp., U.S.A.). Cathode: 2 cm × 2.5 cm with surface area of 10 $cm^2$; anode: 1 cm × 3 cm with surface area of 6 $cm^2$. An Ag/AgCl reference electrode (SE11, Xylem Analytics Germany Sales GmbH & Co. KG Sensortechnik Meinsberg, Germany) was pierced through a chloroprene stopper (Deutsch & Neumann GmbH, Germany) and connected to the cathode chamber. The anode chamber of two-chamber reactors was a 50 mL tailor-made glass tube inserted into the four-neck round-bottom flask and ionically connected to the cathode chamber via a cation exchange membrane (CEM, fumasep®FKE, FuMA-Tech GmbH, Germany) fixed by an O-ring and an aluminum cap. It was filled with 40 mL of MSM (without sulfate). All reactors were operated at 30°C and stirred at 400 rpm using a magnetic stirrer. Cathodes were poised at -0.8 V which was chosen according to the preferred potential for hydrogen-mediated sulfate reduction [33] and

current was recorded every 600 s using a multipotentiostat (MPG-2, Bio-Logic Science Instruments, France).

## 2.4. Microcosm experiments

Microcosm experiments were performed in 100 mL serum bottles containing 45 mL MSM with 10 mM sulfate being flushed with a $CO_2/N_2$ mixture (20 %:80 %, v/v) for 30 min. In accordance to the respective experiments, 0.5 bar of $H_2$ or $O_2$ were supplemented to the serum bottles. The microcosm experiments were inoculated with 5 mL of the reactor liquid obtained from the two-chamber reactors after the BES experiment was finished. For inhibiting SRP activities, 0.5 mL of an anoxic sodium molybdate stock solution (1 M), yielding a final concentration of 10 mM, were added to microcosm experiments [34]. All microcosm experiments (Table 2) were performed in triplicates and incubated at 30 °C under static conditions.

## 2.5. Chemical analyses

For liquid sampling, a needle was pierced through the rubber using a 5 mL syringe beforehand flushed with nitrogen. First, 5 mL of reactor liquid was discarded and then 5 mL of sample was obtained. Therefrom, 4 mL of liquid was directly deep frozen in liquid nitrogen and stored at -80 °C. From the remaining liquid, 0.2 mL were directly fixed by adding 1 mL of zinc acetate solution (3 %) for soluble sulfide measurement via a modified methylene blue method which was described previously [35]. The remaining 0.8 mL were used for $OD_{600}$ and pH measurements. The pH was measured via a pH meter (SevenCompact S220, Mettler Toledo GmbH, Germany). The $OD_{600}$ was measured via a spectrophotometer (U-2000, Hitachi High-Tech Corporation, Japan) by using MSM as blank.

Deep frozen samples were gently melted within an anaerobic glovebox and fixed by adding 1 mL 100 mM zinc acetate solution to fix soluble sulfide. The well-mixed solution was filtered using a 0.2 µm PTFE filter (VWR®, U.S.A.). Subsequently, sulfate was quantified by ion chromatography (IC) with a conductivity detector (Suppressed conductivity, ADRS 600, 2 mm) using a Dionex ICS-6000 equipment, an IonPac AG18 column (2 mm, Thermo Fisher Scientific Inc., U.S.A.) by using potassium hydroxide as eluent at 30 °C with a flow rate of 0.25 mL $min^{-1}$ for 14 min.

Samples for acetate analysis were taken after the experiments. 2 mL samples were centrifuged at 10,000 g for 10 min and filtered with a 0.2 µm PTFE filter. The samples were analyzed by using high-performance liquid chromatography (HPLC, Shimadzu Scientific Instruments, Japan) equipped with a photodiode array detector (SPD-M20A prominence, Shimadzu Scientific Instruments, Japan), a Hi-Plex H column (300 mm × 7.7 mm ID, 8 µm pore size, Agilent Technologies, U.S.A.), and a pre-column (Carbo-H 4 mm × 3 mm ID, Security Guard, Phenomenex, U.S.A.) eluted by 5 mM $H_2SO_4$ at 65 °C with a flow rate of 0.6 mL $min^{-1}$ for 30 min.

## 2.6. Microbial community analysis

Microbial samples were taken at the end of each experiment. The biofilm samples from BES experiments were obtained with a sterile spatula, while planktonic cells were harvested by centrifuging 16 ml reactor liquid (10 min, 4°C, 10000 g). Both, pellet and biofilm, were stored at -30°C until analysis. Genomic DNA from biofilms and planktonic cells was extracted using the NucleoSpin® kit for soil (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instruction. Fluorometric quantification of extracted DNA was performed using a Invitrogen™ Qubit™ Fluorometer and Qubit™ dsDNA HS Assay (Thermo Fisher Scientific Inc., U.S.A.) according to manufacturer instructions.

For sequencing, 16S rRNA genes were amplified using the primer-set 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') targeting the V4 variable region [36]. The 25 µL of PCR mixture contained 12.5 µL Mytaq$^{TM}$ HS Mix 2x (Bioline, UK), 0.2 to 43.9 ng of template DNA, 1 µL of BSA (1:20), 1 pM of each primer, and PCR-grade $H_2O$ to a final volume of 25 µl. The PCR mixture was initially denatured for 3 min at 98°C, followed by a total of 35 cycles (with each including 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C), with a final extension step at 72°C for 5 min. Each sample was amplified in two replicates. Before sequencing on Illumina's MiSeq platform (paired-end, 2x250 reads), library preparation was performed according to manufacturer's instructions using NexteraXT kit for indexing. Qiime2 (version 2021.2) [37] with cutadapt (version 1.15) [38], DADA2 (1.18.0) [39], fastQC (0.11.5, http://www.bioinformatics.babraham.ac.uk/projects/fastqc), multiQC (1.10.1) [40], and SILVA release 138 [41] were used for trimming, quality control, and taxonomic assignment of raw and de-multiplexed sequence reads. For final analysis using phyloseq in R [42], the replicate with the highest amount of reads was chosen and sequence reads were transformed to relative abundance to determine the diversity at phylum and order level for estimating prevalent genera.

## 2.7. Calculations

The electron recovery in terms of sulfate reduction ($ER_{\text{sulfate}}$) was calculated by Eq. 2:

$$ER_{\text{sulfate}} = \frac{zFV(C_0 - C_1)}{\int I\, dt} \qquad \text{(Eq. 2)}$$

*I* is current, *t* is time, *z* is the number of electrons (8 electrons for complete reduction of sulfate to sulfide according to Eq. 1), *F* is the Faraday constant (96,485.3 C mol$^{-1}$), *V* is working volume of

193 the reactors (250 mL), $C_0$ and $C_1$ are the sulfate concentrations at the beginning and the end of the
194 experiment of each reactor, respectively.

195 The redox potential of the oxygen evolution reaction ($E_{\mathrm{OER}}$) in aqueous solution mainly depends
196 on the pH of the solution and was calculated according to Eq. 3 and 4:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad \text{(Eq. 3)}$$

197

$$E_{\mathrm{OER}} = E^0 + \frac{RT}{F} ln(\mathrm{pH}) \qquad \text{(Eq. 4)}$$

198

199 The standard Gibbs free energy of reaction of the aerobic oxidation of bisulfide to sulfate ($\Delta_f G^{0\prime}_{\mathrm{sul1}}$)
200 was calculated using tabulated values [43] (Eq. 5):

$$\mathrm{HS^-} + 2\mathrm{O_2} \rightarrow \mathrm{SO_4^{2-}} + \mathrm{H^+} \qquad \Delta G^{0\prime}_{sul1} = -796\ kJ\ mol^{-1} \qquad \text{(Eq. 5)}$$

201

202 The standard Gibbs free energy of reaction of the anaerobic electrochemical oxidation of bisulfide
203 to sulfate ($\Delta_f G^{0\prime}_{\mathrm{sul2}}$) was calculated using tabulated values [43] (Eq. 6). Please note that it is required
204 to establish a reduction reaction for obtaining redox potentials from Gibbs free energy calculations
205 [44].

$$\mathrm{SO_4^{2-}} + 9\mathrm{H^+} + 8\mathrm{e^-} \rightarrow \mathrm{HS^-} + 4\mathrm{H_2O} \qquad \Delta G^{0\prime}_{sul2} = 167\ kJ\ mol^{-1} \qquad \text{(Eq. 6)}$$

206

207 Subsequently, $\Delta_R G^{0\prime}_{\mathrm{sul2}}$ was corrected for non-standard conditions via Eq. 7 yielding $\Delta_R G_{\mathrm{sul2}}$ and
208 the corresponding redox potential ($E_{\mathrm{sul2}}$) was calculated by applying Eq. 8.

$$\Delta_R G_{sul2} = \Delta_R G^{0\prime}_{sul} + RTln\left[\left(\frac{C_{HS-}}{C^0_{HS-}}\right) \times \left(\frac{C^0_{SO42-}}{C_{SO42-}}\right) \times \left(\frac{C^0_{H+}}{C_{H+}}\right)^9\right] \quad \text{(Eq. 7)}$$

209

$$E_{sul2} = \frac{\Delta_R G_{sul2}}{z \times F} \quad \text{(Eq. 8)}$$

210

211 $R$ is the gas constant (8.314 J mol$^{-1}$ K$^{-1}$), $T$ is temperature, $C_i$ is concentration of i-th species, $C_i^0$
212 is standard concentration of i-th species (1 M, for $C_{H+}$ $10^{-7}$ M).

213 2.8. Statistics and experimental replicates

214 All biotic experiments were performed in at least triplicates enabling calculations of mean values
215 and standard deviations. Detailed information on replicates and experimental parameters of all
216 experiments is shown in Table 2.

217 Table 2 Overview on experiments conducted in this study.

| Experiment | Short description | Replicates |
|---|---|---|
| BES experiments | | |
| One-chamber reactor | Cathodic $H_2$ and anodic $O_2$ were provided by water electrolysis to the microorganisms | 3 |
| Two-chamber reactor | Only cathodic $H_2$ was provided by water electrolysis to the | 4 |

| | | |
|---|---|---|
| | microorganisms(the anode is separated by a membrane) | |
| Abiotic one-chamber reactor | Cathodic $H_2$ and anodic $O_2$ were provided by water electrolysis, no microorganisms | 1 |
| Abiotic two-chamber reactor | Only cathodic $H_2$ was provided by water electrolysis (the anode is separated by a membrane), no microorganisms | 1 |
| Microcosm experiments | | |
| Anoxic microcosm, $H_2$ was added | | 3 |
| Anoxic microcosm, $H_2$ and molybdate were added | Inoculated with reactor liquid from two-chamber reactor | 3 |
| Anoxic microcosm, no $H_2$ was added | | 3 |
| Aerobic sulfide oxidizing microcosm, biotic | Inoculated with the same inoculum used for BES experiments; sulfide and oxygen were added. | 3 |
| Aerobic sulfide oxidizing microcosm, abiotic | No inoculum, sulfide and oxygen were added. | 3 |

218

## 3. Results

### 3.1. Characterization of bioelectrochemical systems

Figure 1 shows the chronoamperograms and the concentrations of sulfate and sulfide for one- and two-chamber reactors (cathode potential -0.8 V, initial sulfate concentration of 10.84 ±0.64 mM). Both reactor configurations had an initial current density (*j*) of approximately -0.2 mA $cm^{-2}$. Whereas *j* of one-chamber reactors decreased to -0.47 ±0.13 mA $cm^{-2}$, *j* of two-chamber reactors stabilized at -0.07 ±0.02 mA $cm^{-2}$ during the duration of the experiment.

At the end of the experiment, the sulfate removal efficiency and the electron recovery ($ER_{sulfate}$) in two-chamber reactors reached 78.3 ±16.4 % and 83.9 ±1.3 %, respectively, being higher than most studies reporting a maximum of 70 % sulfate removal and an electron recovery of around 50 % during batch operation (Table 1) [30, 45]. As the sulfide concentration in the catholyte of two-chamber reactors increased to 6.84 ±1.06 mM at the end of the experiment, the sulfate-sulfide-balance reached 91.1 ±8.0 % indicating only minor sulfide loss due to $H_2S$ volatilization and precipitation as, for instance, FeS [46]. This is in line with literature reporting a maximum 88 % sulfur balance [23]. In contrast, no sulfate removal was observed in one-chamber reactors, and the sulfide concentration reached only 0.05 ±0.03 mM during the reactor operation. Corresponding abiotic reactors using the identical operation conditions did not show sulfate removal (Fig. S3).

After inoculation, $OD_{600}$ increased in one- and two-chamber reactors indicating growth of planktonic microorganisms (Fig. S1). After ca. 5 days, $OD_{600}$ remained stable at around 0.1 for both reactor configurations until the end of the experiment. Notably, black precipitates accumulated on the surface of cathode together with microorganisms in two-chamber reactors, whereas neither precipitates nor biofilms were observed on the surface of cathodes in one-chamber

241 reactors (Fig. S2). Nevertheless, biomass could be extracted from the cathodes of both reactor
242 configurations (see section 3.3). The pH in one-chamber reactors was nearly constant at 7.3 ±0.2,
243 whereas the pH of the catholyte of the two-chamber reactors increased from 7.1 ±0.0 to 8.5 ±0.1
244 at the end of the experiment (Fig. S1).

245 In summary, the BES performance data showed clearly that despite of identical operational
246 conditions, microbial electrochemical sulfate reduction only occured in two-chamber reactors but
247 not in one-chamber reactors. This was surprising as the comparable *j* in one-chamber reactors
248 indicated a high availability of $H_2$ representing a suitable electron donor for sulfate reduction [11].

249 [Please insert Figure 1]

250 3.2. Elucidating the role of the reactor configuration on the microbial electrochemical sulfate
251 reduction using non-electrochemical microcosm experiments

252 To elucidate the role of the reactor configuration and thus of anodically produced $O_2$ on sulfate
253 removal, non-electrochemical microcosm experiments were performed (i.e., sulfate microcosm
254 experiment, SME). Therefore, these microcosm experiments were inoculated with planktonic
255 cultures derived from two-chamber reactors and oxygen was regularly added.

256 In the presence of sulfide and oxygen in the microcosm experiments, sulfide was oxidized in both
257 abiotic and biotic cultures at a similar rate. This was confirmed by regularly adding sulfide (Fig.
258 S4). Therefore, one likely explanation for the observed net zero microbial electrochemical sulfate
259 reduction in one-chamber BES is the instantaneous abiotic re-oxidation of sulfide by anodically
260 formed $O_2$ presumably leading to an internal sulfur cycle. This was supported by basic
261 thermodynamic calculations using time-resolved analytical and electrochemical data. The anode
262 potential of one-chamber reactors and the redox potential for the OER were 2.07 ±0.10 V and 0.80

±0.01 V (considering only pH, see Eq. 4), respectively. Considering also that the sulfide oxidation by $O_2$ as oxidant is strongly exergonic (Eq. 5), anodic oxygen production and consequently abiotic sulfide oxidation is highly feasible. In contrast, biotic sulfide oxidation did not seem likely (Fig. S4). Furthermore, also abiotic electrochemical sulfide oxidation is thermodynamically feasible considering its redox potential corrected for actual reaction conditions (-0.21 ±0.02 V, Fig. S5) and the observed anode potential.

### 3.3 Investigating the mode of cathodic electron transfer

Further non-electrochemical microcosm experiments were performed for verifying that $H_2$ is the main electron source for sulfate reduction in experiments with BES. Therefore, the microcosm experiments were inoculated with planktonic cultures derived from two-chamber reactors and, depending on the experiment, $H_2$ and $H_2$+molybdate were added. Experiments without $H_2$ and with $H_2$+molybdate, which is an inhibitor of SRP [34], in the presence of sulfate resulted in no sulfate reduction (Fig. 2). In contrast, by adding hydrogen and sulfate to the microcosm experiments, complete sulfate reduction was achieved within approx. 15 d with no apparent lag phase (Fig. 2). This indicated a key role of hydrogenotrophic SRP on sulfate reduction during chronoamperometric cultivation in BES experiments. Interestingly, acetate was detected at the end of microcosm experiment with a concentration of 0.63 ± 0.07 mM and 10.14 ± 0.04 mM when $H_2$ and $H_2$ +molybdate were present, respectively, indicating that $H_2$ was utilized in both microcosm experiments by homoacetogens producing acetate from $H_2$ and $CO_2$. No acetate was detected in the control microcosm experiment (without $H_2$) providing evidence that hydrogen is necessary for acetate formation.

[Please insert Figure 2]

### 3.3. Microbial community analysis

In total, 3,260,597 reads were gathered from all samples obtained from BES and microcosm experiments. Reads per sample ranged from 22,241 to 138,348. Shannon diversity ranged from 3.5 to 5.5 with communities of the one-chamber reactors being less diverse than communities of the two-chamber reactors being less diverse than communities of microcosm experiments. As shown in Fig. 3a, the most abundant phyla found in the inoculum were *Desulfobacterota* and *Firmicutes*. The most abundant phyla in microbial samples derived from two-chamber reactors, and microcosm experiments with/without $H_2$ was *Desulfobacterota*. In contrast, microcosm experiments with $H_2$+molybdate and with $O_2$ were dominated by the phylum *Firmicutes*. In one-chamber reactors, *Proteobacteria* were most abundant which were also present in the inoculum. In one- and two-chamber reactors, the microbial composition was similar for biofilms and planktonic cultures.

At genus level, the inoculum used for the chronoamperometric cultivation in BES contained 21.7 ±3.1 % SRP consisting of *Desulfomicrobium*, *Desulfovibrio*, *Desulfobulbus*, and *Desulfococcus* as major taxa (Fig. 3b). The two SRP *Desulfomicrobium* and *Desulfovibrio* were considerably enriched with an abundance of 73.8 ±0.2 % and 49.9 ±11.7 % in the cathode biofilm and planktonic culture, respectively, of two-chamber reactors. *Desulfomicrobium* has been numerous times observed to use either organics and/or $H_2$ as electron donor for reducing sulfate and other inorganic sulfur species [47, 48]. Similarly, *Desulfovibrio* is well-studied, capable of oxidizing $H_2$ [49], and has been often described in sulfate-reducing systems and in microbial electrochemical sulfate reduction [22, 23, 50]. In contrast, SRP in one-chamber reactors only accounted for 0.2 ± 0.2 % and 1.6 ± 1.6 % in cathode biofilm and planktonic culture, respectively, demonstrating a minor role within the determined microbial community. Presumably, growth of

SRP was inhibited in one-chamber reactors by anodically produced $O_2$ (see section 3.2). The most abundant genus in one-chamber reactors was *Acinetobacter* accounting for 76.3 ±0.1 % and 27.5 ±35.8 % in the cathode biofilm and planktonic culture, respectively. *Acinetobacter* was identified in the environmental sulfur cycle [51] and related to bacteria being capable of sulfide oxidation [52].

In microcosm experiments with $H_2$, the most abundant genera were *Desulfomicrobium* and *Desulfovibrio* with abundances of 53.4 ±2.8 % and 33.9 ±4.0 %, respectively, confirming results of two-chamber reactors (Fig. 3b). In addition, low abundances of the mainly homoacetogenic genera *Acetobacterium* (0.4 ±0.0 %) and *Acetoanaerobium* (0.8 ±0.1 %) were observed reflecting the low acetate concentration detected in these experiment. In contrast, *Acetobacterium* dominated the microbial community in the microcosm experiments with $H_2$+molybdate by 53.2 ±7.8 % offering an explanation for the observed high acetate concentration at the end of incubation (10.14 ±0.04 mM) [53, 54].

[Please insert Figure 3]

## 4. Discussion

By applying a cathode potential of -0.8 V (vs. SHE), the HER was promoted generating sufficient $H_2$ for achieving a chemolithoautotrophic sulfate reduction. A sulfate reduction rate of 9.7 ±2.6 mg $L^{-1}$ $d^{-1}$ $cm^{-2}$ was observed in two-chamber reactors which is comparable to previously reported values [28, 55]. At the same time, the determined sulfate removal efficiency of 78.3 ±16.4 % and $ER_{sulfate}$ of 83.9 ±1.3 %, are higher than usually reported values (max. 70 % sulfate removal and 50-80 % $ER_{sulfate}$, Table 2 and [56, 57]). Nevertheless, in several studies on microbial electrochemical sulfate reduction, additional electron and carbon sources (e.g., ethanol and acetate)

were added challenging a consequent assessment of $ER_{sulfate}$. This is even undermining the advantage of MET that only electric energy is needed and that addition of chemicals is not required [32, 58, 59]. Presumably, sulfate reduction was mainly performed by *Desulfomicrobium* and *Desulfovibrio* as they represented the most dominant genera in two-chamber reactors. Notably, the abundance of both genera was higher in the cathode biofilm than in the planktonic culture (Fig. 3) indicating electrotrophic and/or hydrogenotrophic pathways for sulfate reduction. *Desulfovibrio* was reported to be able to directly accept electrons from cathodes [60] but also $H_2$ consumption for sulfate reduction is described [61, 62]. However, it is also described that most *Desulfovibrio* species need small amounts of acetate for hydrogenotrophic growth [4]. Similarly, *Desulfomicrobium* were reported as SRP consuming organic substrates representing a heterotrophic pathway [63] for sulfate reduction. Interestingly, the homoacetogenic genera *Acetobacterium* and *Acetoanaerobium* were also be identified in two-chamber reactors, even though with a low abundance (0.7 ±0.3 % and 0.07 ±0.02 %, respectively). Therefore, they potentially provided low amounts of acetate for SRP from $CO_2$ and cathodically produced $H_2$ that was also indicated by corresponding microcosm experiments. Similar syntrophic relationships were already described in $H_2$-amended sulfate-reducing reactor systems [64].

[Please insert Figure 4]

However, no net sulfate reduction and no sulfide formation were observed in one-chamber reactors being contradictory to previous studies describing sulfate removal therein [28, 31]. One important difference compared to the present work, applying initially chemolithoautotrophic conditions, is the addition of ethanol as electron donor and carbon source representing a further metabolic opportunity for SRP [31, 65]. Guan and colleagues achieved sulfate removal in one-chamber reactors using *Desulfovibrio caledoniensis* and a steel cathode poised to -0.6 V [28, 61]. We

speculate that metal sulfides are formed at this material that cannot be re-oxidized leading to an apparent sulfate removal [46].

For further shedding light on the experimental observations and for deciphering the most probable pathways occurring in BES, microcosm experiments were performed. As a result, the high likelihood of hydrogentrophic sulfate reduction in two-chamber reactors was demonstrated (Fig. 4b). However, the high concentrations of acetate and the high abundance of homoacetogens (53.2 ±7.7 % *Acetobacterium* and 1.4 ±0.4 % *Acetoanaerobium*) in the $H_2$+molybdate experiments indicate that excess $H_2$ in two-chamber reactors was utilized for acetate production enabling also mixotrophic sulfate reduction although initially fully chemolithoautotrophic conditions were applied.

In contrast to two-chamber reactors, the reaction conditions in one-chamber reactors were more complex potentially facilitating different reaction pathways. Obviously, the lack of a membrane resulted in crossover of anodically produced $O_2$ presumably inhibiting strictly anaerobic SRP. Therefore, the cathodically produced $H_2$ could be used by hydrogenotrophs as also indicated by the presence of *Acenitobacter* and *Pseudoxanthomonas* in cathode biofilms of one-chamber reactors [66, 67]. Subsequently, acetate and also cathodically produced $H_2$ were consumed by mixotrophs like, for instance, *Campilobacterota* [68] and *Proteobacteria* (Fig. 4b). Therefore, this metabolic short circuit further prevented cathodic sulfate reduction by its superior growth rate. Another type of chemical short circuit that potentially occurred is the abiotic hydrogen oxidation at the anode supported by the observed anode potential of 2.07 ± 0.10 V. Nevertheless, the traces of sulfide observed in one-chamber reactors (Fig. 1A) indicates also the presence of cathodic sulfate reduction either with a negligible rate or accompanied by simultaneous re-oxidation of sulfide to sulfate representing an internal sulfate-sulfide-cycle. Here, abiotic processes seems more

likely. Sulfide could be oxidized i) electrochemically directly at the anode (Fig. 4a) also supported by thermodynamic calculations (Fig. S5), or ii) by anodically produced oxygen (Fig. 4a). Although an internal sulfate-sulfide-cycle cannot completely ruled out, yet we speculate that it probably has a rather minor influence on the not observed sulfate reduction in one-chamber reactors. Instead, a chemical short circuit including cathodically produced hydrogen and anodically produced oxygen seems more likely considering that electrochemical reactions usually exhibit superior rates compared to microbiological reactions [69].

Although the experimental setup was designed for performing fundamental studies on cathodic sulfate reduction, the obtained rates are comparable to more application-oriented studies and the electron recovery is even higher [22, 29, 56, 57]. Nevertheless, volumetric rates and capex offer substantial room for improvements by engineering of electrodes and reactor design. For instance, bed reactors represent a cost-effective, easy-to-use, and robust opportunity for a plethora of applications especially for a controllable removing of pollutants [70, 71]. From an application-oriented perspective, it is of note that the performed experiments did not required addition of organics for sulfate reduction. Instead, BES were operated at chemolithoautotrophic conditions representing a substantial advantage in terms of opex when compared to many previously reported BES studies on sulfate reduction and non-electrochemical bioremediation.

Furthermore, it is worth noting that the carefully indicated cryptic sulfur cycle, whereby sulfide (upon production from SRP) is rapidly back-oxidized to sulfate at the anode of one-chamber reactors in presence of anodically produced $O_2$, has important implications in the implementation of novel electrobioremediation technologies [72]. Although the results do not provide clear evidence about the importance of this cycle to the overall process, it can be assumed that it is only of minor importance in the studied experimental system. Indeed, in certain anaerobic ecosystems

such as petroleum hydrocarbons-contaminated groundwaters or marine sediments, sulfate-reduction is often the dominant respiratory process [73]. In such environments, the oxidation of contaminants is often rate-limited by the availability of naturally occurring sulfate and/or other electron acceptors. In this context, providing a polarized anode would represent an effective strategy to continuously regenerate sulfate (i.e., the electron acceptors) whilst being reduced in cathode, thereby sustaining a more effective bioremediation. Though the occurrence of this process has been suggested to play a role in previous studies whereby an anode buried within the contaminated matrix was found to accelerate hydrocarbons oxidation under sulfate-reducing conditions, no experimental evidence for a complete (bio)electrochemical sulfide oxidation to sulfate was ever provided [74-77]. Additionally, besides contributing to sulfate regeneration, the (bio)electrochemical oxidation would alleviate the inhibitory effects possibly caused by the accumulation of high concentration of sulfide [78]. Clearly, further studies are needed to identify the optimal anode potential and material maximizing the rate and efficiency of sulfide oxidation to sulfate, while, for instance, minimizing its conversion into insoluble elemental sulfur.

## 5. Conclusion

The microbial electrochemical sulfate reduction to sulfide was investigated in a comparative study using one- and two-chamber reactors. A sulfate reduction rate and an electron recovery of 9.7 ±2.6 mg $L^{-1}$ $d^{-1}$ $cm^{-2}$ and 83.9 ±1.3 %, respectively, were achieved in two-chamber reactors. Cathodically produced hydrogen represented the main electron source and microbial communities were dominated by typical sulfate-reducing prokaryotes from the genera *Desulfomicrobium* and *Desulfovibrio*. In contrast, no sulfate reduction was observed in one-chamber reactors. Despite several pathways are conceivable for this observation, a chemical short circuit including abiotic and biotic reactions with cathodically produced hydrogen and anodically produced oxygen seems

most plausible. In addition, an internal sulfate-sulfide-cycle between sulfate-reducing microorganisms and abiotic sulfide oxidation could also contributed to this phenomenon. The obtained results strongly suggest to only perform experiments on microbial electrochemical sulfate reduction in two-chamber reactors for excluding counteracting processes.

## 6. Highlights

- Sulfate reduction in one- and two-chamber BES was evaluated
- Two-chamber BES showed 9.7 ±2.6 mg $L^{-1}$ $d^{-1}$ $cm^{-2}$ SRR and 83.9 ±1.3% $ER_{sulfate}$
- Apparently no sulfate reduction was observed in one-chamber BES
- *Desulfomicrobium* and *Desulfovibrio* dominated microbial community in two-chamber BES

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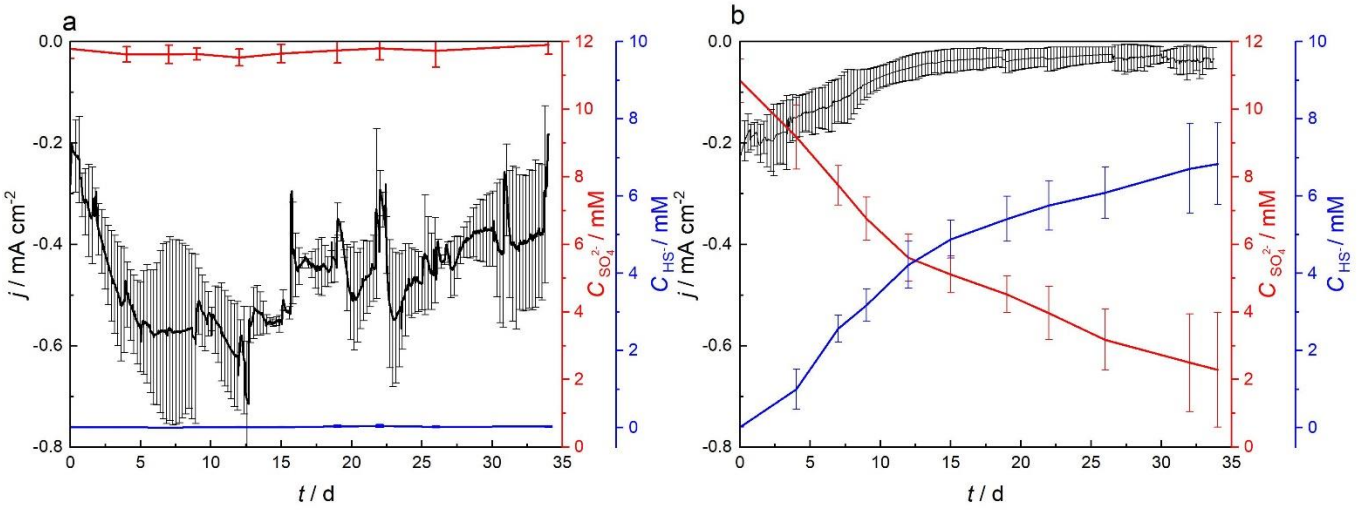


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669 Figure 1

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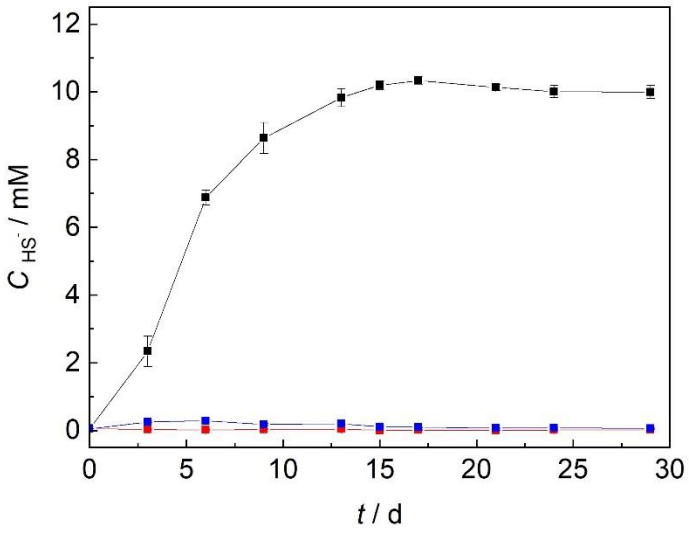


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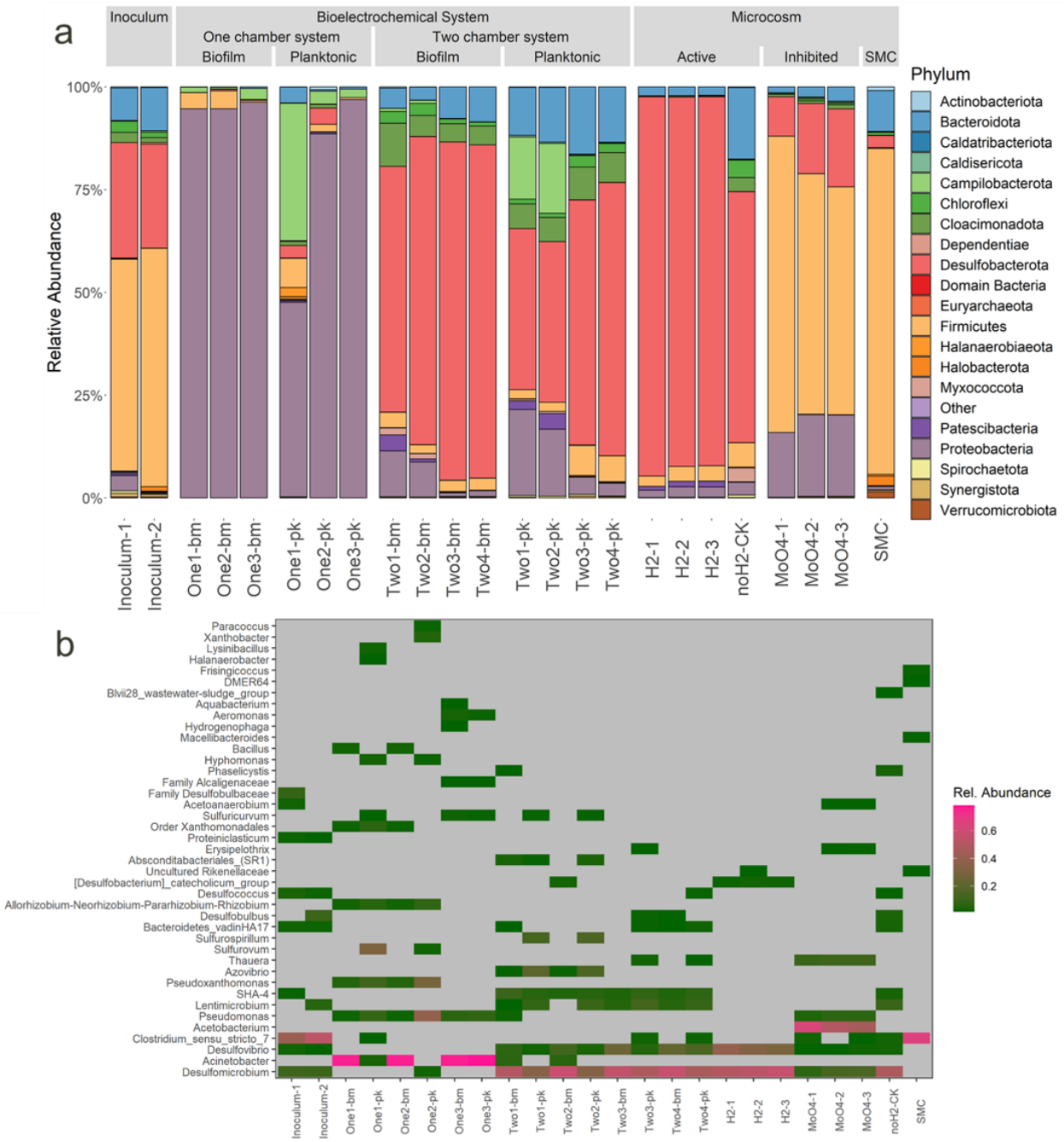


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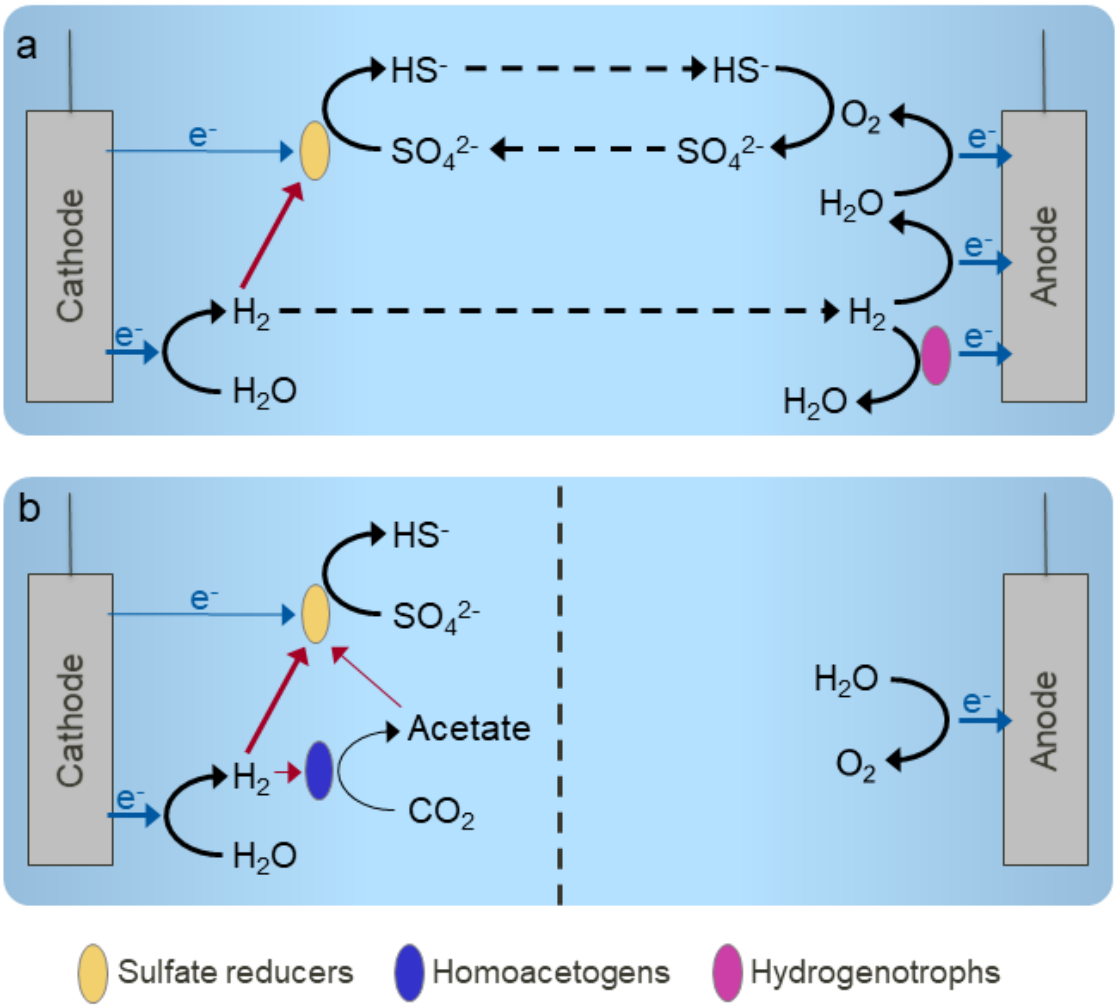


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676 Figure 4

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Fig. 1 Time course of current production (black line), sulfate concentration (red line) and bisulfide concentration (blue line) during chronoamperometric cultivation at -0.8 V using a) one-chamber reactors and b) two-chamber reactors. The error bars represent standard deviations calculated from 3 and 4 replicates for one- and two-chamber reactors, respectively.

Fig. 2 Development of bisulfide concentration in microcosm experiments with sulfate, hydrogen, and molybdate inoculated with planktonic cultures derived from two-chamber BES. The culture supplemented with hydrogen was shown in black, the culture supplemented with hydrogen and molybdate was shown in red, and the culture without hydrogen as control was shown in blue. The error bars represent the standard deviations (n=3).

Fig. 3 Taxonomic classification of the dominant phylogenetic groups of microbial samples from reactors and microcosm cultures at a) phylum level and b) genus level with the number indexing the replicate. The inoculum are the sulfate-reducing inoculum described in SI 1 used for bioelectrochemical systems as well as the sulfide microcosm culture (SMC). One-/ Two-bm/pk are the microbial samples from cathode biofilm / planktonic culture from one-/two-chamber reactors, respectively. H2, MoO4, and noH2 represent the microbial samples from microcosm culture that are supplemented with $H_2$, $H_2$ together with molybdate and no $H_2$ nor molybdate which were inoculated with the reactor liquid from two-chamber reactors. SMC is the sulfide microcosm culture supplemented with $O_2$ inoculated with sulfate-reducing inoculum. The microcosm culture of noH2 and SMC are the combined samples from all the three replicates due to low biomass concentration.

699 Figure 4 Illustration of the presumed reaction pathways of sulfate in one- and two-chamber
700 reactors: a) In one-chamber reactors, the electron sources for sulfate reduction to bisulfide are
701 mainly cathodically produced hydrogen and only to a minor extent immediate microbial electron
702 uptake from the cathode. Subsequently, bisulfide its re-oxidized by anodically produced oxygen
703 leading to no apparent sulfate reduction. Furthermore, a considerable share of the cathodically
704 produced hydrogen is biotically oxidized by hydrogenotrophs or abiotically oxidized at the anode.
705 b) In two-chamber reactors, sulfate reducers utilize cathodically produced hydrogen and received
706 electrons as electron donors for reducing sulfate. Hydrogen is also used by homoacetogens for
707 reducing carbon dioxide to acetate that serves as additional carbon source and electron donor for
708 sulfate reducers. The integrated membrane in two-chamber reactors decrease crossover of sulfur
709 species, oxygen, and hydrogen preventing bisulfide re-oxidation and material short circuits. The
710 blue, red, and black arrows indicate electron transfer, tranport processes, and reactions,
711 respectively.

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