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Microbial community analysis in a long-term membrane-less microbial electrolysis cell with hydrogen and methane production

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2-bromoethanesulfonate (BES), hydrogen, methane, microbial electrolysis cell (MEC), pyrosequencing, qPCR

ABSTRACT
A single-chamber microbial electrolysis cell (MEC) aiming at hydrogen production with acetate as sole carbon source failed due to methanogenesis build-up despite the significant amount of 2-bromoethanesulfonate (BES) dosage, 50 mM. Specific batch experiments and a thorough microbial community analysis, pyrosequencing and qPCR, of cathode, anode and medium were performed to understand these observations. The experimental data rebuts different hypothesis and shows that methanogenesis at high BES concentration was likely due to the capacity of some \textit{Archaea} (hydrogen-oxidizing genus \textit{Methanobrevibacter}) to resist high BES concentration up to 200 mM. \textit{Methanobrevibacter}, of the \textit{Methanobacterales} order, represented almost the 98\% of the total \textit{Archaea} in the cathode whereas \textit{Geobacter} was highly abundant in the anode (72\% of bacteria). Moreover, at higher BES concentration (up to 200 mM), methanogenesis activity decreased resulting in an increase of homoacetogenic activity, which challenged the performance of the MEC for H\textsubscript{2} production.
1. INTRODUCTION
The need of renewable energy resources is an important focus of current research. Hydrogen gas (H\(_2\)) is a preferred alternative energy source since it is a clean and renewable energy carrier, without an impact on the greenhouse gas emission during its energy generation step and a high combustion heat (120 kJ/g) when compared to other possible biofuels (CH\(_4\), 50 kJ/g or ethanol, 26.8 kJ/g) [1]. However, nowadays, most H\(_2\) is produced via steam reforming, a non-sustainable option. Among all the current biological H\(_2\) production techniques, the utilization of bioelectrochemical systems is very attractive because high yields can be achieved. Dark fermentation would only produce a maximum amount of 4 moles of H\(_2\) per mole of glucose while most of the electronic content in substrate, except for the growth requirements, could be recovered using bioelectrochemical systems [2].

H\(_2\) production in bioelectrochemical systems is conducted in devices known as microbial electrolysis cells (MECs). MECs use the particular biochemical pathway of a group of bacteria named exoelectrogens, which are able to transfer the electrons gained in their metabolism out of the cell and use a solid as electron acceptor. These electrons flow from the anode to the cathode where they react with protons to produce H\(_2\). These protons are generated in the anodic oxidation process and are transported from the anode to the cathode through the electrolyte. Hence, exoelectrogenic bacteria act as biocatalysts of the oxidation process enabling H\(_2\) production. Fundamental thermodynamics indicate that the H\(_2\) production process in an MEC is not spontaneous. Thus, an additional voltage must be applied depending on the organic substrate used. For example, 0.14 V (under standard biological conditions according to the Nernst equation) should be enough for H\(_2\) production from acetate [3].

The truth is that, in practice, a higher applied voltage (0.5 to 1 V) is needed under lab conditions due to the high potential losses [4]. Among all the voltage losses of the cell, the losses over the membrane, when used, are very significant. Membranes provide a separation between anode and cathode, which prevents H\(_2\) from being used by H\(_2\) scavengers and avoids impurities in H\(_2\) [5]. Thus, either membrane losses are reduced with the development of new materials or the membrane itself is suppressed (i.e. single-chamber systems). Call et al. [6] questioned for the first time the necessity of membranes in single-cell MECs since: i) MECs do not need oxygen as microbial fuel cells (MFCs) and hence oxygen leakage from the cathode to the anode is not possible; ii) H\(_2\) has a very low solubility and should be scarcely used by microorganisms in the cell and therefore could be mostly recovered; iii) current densities should not be decreased because of the membrane absence, and iv) the possible H\(_2\) losses are compensated by the reduced cost of the system in terms of materials and potential applied. Since this work, many MEC studies have been conducted with and without membrane. Regarding membrane-less MECs, its success is challenged by the bacteria colonizing each electrode. The anode is mostly colonized by exoelectrogenic bacteria (usually from the genera Geobacter and Shewanella). Geobacter is the most dominant genera in acetate-fed MEC [7]. Nevertheless, MEC conditions (i.e. anaerobic environments with organic matter as electron donor) may enable the proliferation of methanogens. Methanogens competing against the exoelectrogens for the same substrate or using the H\(_2\) produced in the cathode is one of the most important reported failures of MECs [8,9]. Thus, understanding and inhibiting methanogenesis in these systems is essential in view of its future implementation. The use of a chemical inhibitor, 2-bromoethanesulfonate (BES) has been proposed as a suitable option for methanogenesis prevention in different anaerobic research fields [10]. BES is a structural analog of Coenzyme M (CoM) and inhibits effectively the methyl-CoM reductase reaction [11–13]. As abovementioned, methanogenesis can be seen as a competition of bioelectrochemical H\(_2\) production but also as an opportunity if methane (CH\(_4\)) production linked to H\(_2\) production cannot be avoided. Producing CH\(_4\) rather than H\(_2\) gas may be the best
Methanogens can produce CH\textsubscript{4} from CO\textsubscript{2} and H\textsubscript{2} but also methanogens that can directly use electrons from the cathode to produce CH\textsubscript{4}, i.e. electromethanogenesis, have been reported [14].

The objective of this study is to gain understanding on the biological processes occurring in the cathode of a membrane-less MEC aiming at H\textsubscript{2} production, through an engineering approach and a thorough microbial analysis. Several BES concentrations to limit methanogenesis are tested to elucidate the fate of H\textsubscript{2} under different scenarios.

2. MATERIALS AND METHODS

2.1. Reactor description and operation

A large single-chamber membrane-less MEC (1300 mL) was used to carry out the experiments. A carbon fiber brush (PANEX®33 160 K, ZOLTEK) [15] previously inoculated in an MFC, as previously described [16], was used as anode (0.8 m\textsuperscript{2}). The cathode (0.034 m\textsuperscript{2}) was made with carbon cloth coated with carbon powder and platinum suspension on the side facing the anode [17]. Both electrodes were arranged concentrically with the cathode in the outer perimeter, so that all ends of the anode were at the same distance from the cathode. The reactor operated in batch mode, with constant agitation and an applied potential of 1.2 V between anode and cathode (HQ Power, PS-23023). The medium used (pH 7.3) was a 100 mM phosphate buffer with acetate (12 mM) as substrate and the following components in 1 L of deionized water: NH\textsubscript{4}Cl (0.41 g), mineral media (5 mL), 1 mL of 4 g·L\textsuperscript{-1} FeCl\textsubscript{2} stock solution, and 0.5 mL of 37.2 g·L\textsuperscript{-1} Na\textsubscript{2}S·9H\textsubscript{2}O stock solution. The mineral medium had the composition previously described in Parameswaran et al. [18]. MEC was operated with the addition of BES (Sigma-Aldrich, USA). BES (Br-CH\textsubscript{2}CH\textsubscript{2}-SO\textsubscript{3}Na) is a sulfonate composed of a sulfite group (SO\textsubscript{3}\textsuperscript{2-}) and an ethyl group (C\textsubscript{2}H\textsubscript{5}-) with one H substituted by a bromine (Br) atom. The MEC was inoculated and operated for 4 months using 50 mM of BES concentration, according with common practice [19]. Afterwards, the medium was replaced twice adding increasing BES concentrations. First, 100 mM of BES concentration was maintained for 15 days (one batch cycle) and finally fresh medium with 200 mM of BES was added and operated for 20 additional days (one interrupted batch cycle). Other details about the equipment and monitoring system are described in Ruiz et al.[20].

2.2. Electrochemical calculations

Coulombic Efficiency (CE) was calculated as in equation 1.

\[
CE = \frac{\text{Coulombs recovered as current intensity}}{\text{Coulombs in substrate}} = \frac{\int_{t_0}^{t_F} I dt}{F \cdot b_{Ac} \cdot V_L \cdot \Delta c \cdot M^{-1}}
\]

where \(t_0\) and \(t_F\) are the initial and final time of an experiment, \(\Delta c\) is the change in acetate concentration during the experiment (g acetate·L\textsuperscript{-1} cell), \(M\) is the molecular weight of acetate (59 g·mol\textsuperscript{-1}), \(b_{Ac}\) is the number of e\textsuperscript{-} transferred per mole of acetate (8 mol e·mol\textsuperscript{-1} acetate), \(F\) is the Faraday’s constant (96485 C·mol\textsuperscript{-1} e\textsuperscript{-}), \(I\) is the current intensity and \(V_L\) is the volume of liquid in the reactor (L).

Cathodic gas recovery (\(r_{CAT}\)) was calculated as in equation 2.

\[
r_{CAT} = \frac{\text{Coulombs in H}_2}{\text{Coulombs recovered as current intensity}} = \frac{V_{F,H2} \cdot 2 \cdot F \cdot V_{m}^{-1}}{\int_{t_0}^{t_F} I dt}
\]

where \(V_{m}\) is the molar gas volume (24.03 L·mol\textsuperscript{-1}) at 20 °C and \(V_{F,H2}\) is the volume of H\textsubscript{2} at the end of the cycle.
2.3. Chemical analyses
Acetate was analyzed by gas chromatography (Agilent Technologies, 7820-A) using a flame ionization detector (FID) with helium as carrier gas. H₂ and CH₄ production were analyzed by the same gas chromatograph using a thermal conductivity detector with argon as carrier gas. CH₄ relative composition was calculated as the ratio of CH₄ with respect the total amount of CH₄ and H₂ (equation 3).

\[
\text{Relative composition } \text{CH}_4 = \frac{\text{Volume } \text{CH}_4}{\text{Volume } \text{H}_2 + \text{Volume } \text{CH}_4} \tag{3}
\]

This ratio did not consider CO₂, but only H₂ and CH₄. CO₂ could not be quantified simultaneously to CH₄ and H₂ because of using argon as carrier gas in the GC. Individual CO₂ analyses with helium as carrier gas were done sporadically and CO₂ concentration was always around 5%.

BES concentration was measured in medium samples that were serially diluted, filtered (0.22 μ) and analyzed with ion chromatography Dionex ICS-2000 (RFIC) with an Ultimate 3000 Autosampler Column Compartment, a column IonPac AS18 and a pre-column IonPac AG18 (ThermoScientific, USA).

2.4. DNA extraction
Samples were obtained from the anode and the cathode at the end of each experimental period with different BES concentrations. The anode graphite fibers were rinsed with 1ml of sterile MilliQ water to remove residues from the growth medium or residues from biofilm and then were cut and combined for DNA extraction. The same process was applied for the cathode carbon cloth. The medium was also sampled at the end of the period with 50 mM of BES. For this, 1.3 L of the MEC medium was centrifuged at 10000g (Beckmann Coulter TM, Avanti J20XP; USA) to remove supernatant. Total DNA was extracted from approximately 0.15 g of samples using a PowerBiofilm DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s instructions. Quality and quantity of the DNA was measured using a NanoDrop® spectrophotometer (ThermoScientific). Moreover, DNA was visualized under UV in a 0.7% gel electrophoresis with TBE 0.5X (Tris-Borate 50 mM; EDTA 0.1 mM; pH 7.5-8).

2.5. Quantitative real-time PCR
Quantitative hydrolysis probes based real-time PCR (qPCR) was used to quantify total Archaea, the hydrogenotrophic methanogen order Methanobacteriales (MBT) and the exoelectrogen proteobacteria Geobacter as a member of the Fe(III)-reducing Geobacteraceae family.
qPCR was performed with a Lightcycler 480 instrument (LC480; Roche) using the corresponding primers and probes previously described [21–23] (Table 1). Each reaction mixture of 20 μL was prepared using the LightCycler 480 Probe Master kit (Roche Diagnostics), primers for Archaea and Methanobacteriales (final concentration 500nM), primers for Geobacter (final concentration 300nM), hydrolysis probes for Archaea and Methanobacteriales (final concentration 200nM), hydrolysis probes for Geobacter (final concentration 100nM), 2X LC480 Probe Master and 2 μl of template DNA. Amplification of Archaea and Methanobacteriales was performed as described by Yu et al. [21] with slight modifications. Reactions were performed in a three-step procedure: predenaturation for 10 min at 94 ºC followed by 45 cycles of 10 s at 94 ºC, 40 s at 60ºC and 1s at 72ºC (fluorescence detection). Similarly, Geobacter was quantified as described in the literature [22,23] with a
small modification: predenaturation for 10 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 60 s at 55°C and 1s at 72°C (fluorescence detection). All DNA templates were analyzed in duplicate.

Quantitative standard curves were generated using a strategy that differs slightly to the described by Ritalahi et al. [24], particularly for Archaea and Methanobacteriales order quantification. For this purpose, M. formicicum and G. sulfurreducens genomic DNA was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). Target 16S rRNA gene sequences were amplified by conventional PCR using the following combination of primers (Table 1): ARC787F and MBT1196R were used to amplify the V5-V6 region (416 base pairs; bp) from M. formicicum, and GEO561F and GEO825R primers were used to amplify the 16S V3-V4 region of G. sulfurreducens (265 bp). Afterwards, amplicon size was checked in 1% gel electrophoresis. PCR products were gel-purified using GFX PCR DNA & Gel Band purification kit (Healthcare) according to the manufacturer’s instruction. Ligation into pGEMT vector (Promega) and transformation into Escherichia coli DH5α was performed following standard procedures. Sequences of the respective cloned fragments were confirmed by sequencing (Macrogen).

“HERE Table 1”

Afterwards, plasmids containing target fragments were linearized using SalI restriction enzyme (New England Biolabs). Fragments were gel-purified and dialyzed. Finally, each linearized plasmid was serially diluted with a 10-fold step ranging from 10² to 10⁷ copies per PCR reaction mix and directly used as template for qPCR with the corresponding primers and probes (Table 1). The quantification cycle (Cq) values determined were plotted against the logarithm of their initial template copy numbers. Each standard curve was conducted in triplicate.

The number of 16SrDNA copies in plasmid DNA was calculated using the previously described equation [24]. Finally, 16SrDNA concentration (copies/mg) of Geobacter, Methanobacteriales and Archaea in the different samples studied was obtained using equation 4.

\[
\text{n}^\circ \text{ copies per mg} = \frac{\text{elution volume[mL] } \times \text{concentration extracted DNA[n g/µL] } \times \text{concentration template DNA[n g/µL] } \times 2 \mu l}{\text{mg of sample}}
\]

All the samples were quantified in triplicate. When necessary, appropriate dilutions of DNA were made to fit Cq values of samples into standard curves.

2.6. Scanning Electron Microscopy

Samples of graphite fiber brush (anode) and carbon cloth (cathode) were collected at the end of the first period with 50mM of BES, fixed with a solution of 2.5% glutaraldehyde (Sigma-Aldrich) and 2% paraformaldehyde (Sigma-Aldrich), and were processed according to conventional electron microscopy methods as previously described [25]. Samples were treated with osmium tetraoxide, dehydrated with ethanol and dried at critical point with carbon dioxide (BAL-TEC CPD030; Bal-Tec). Then, the samples were coated with few nanometers of Au-C (E5000 Sputter Coater) to increase signal detection and visualized on a Scanning Electron Microscope (Hitachi S-70). As a control, unused fiber brush and carbon cloth were examined following a similar procedure.

2.7. High-throughput 16S rRNA gene pyrosequencing
High-throughput 16S rRNA gene pyrosequencing was performed in 454 Titanium FLX system by the Research and Testing Laboratory (RTL, Lubbock, TX) based upon RTL protocols from cathode and anode DNA after 50 mM BES batch cycle. Each DNA sample (20 ng/μL, quality ratio of 1.8) was analyzed with two different assays and primers couples (with an average of 3000 reads/assay): 349F-806R, comprising the V3-V4 regions of the Archaea 16S rRNA gene [26,27] and 338F-907R, comprising the V3-V5 regions of the bacterial 16S rRNA gene [28]. Sequences were checked using Dechipher (Database Enabled Code for ideal Probe Hybridization Employing R) with Decipher’s Find Chimeras web tool to uncover short-length sequences (less than 1000 nucleotides) chimera (http://decipher.cee.wisc.edu/FindChimeras.html; [29]) and sorting and trimming were done using the Pipeline Initial Process at the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (http://rdp.cme.msu.edu/index.jsp; [30]) with the default settings. The RDP Classifier was used to assign 16S rRNA gene sequences to a taxonomical hierarchy with a confidence threshold of 95%, since the DNA sequences were <250 bp [31]. The relative abundance of a given phylogenetic group was calculated as the number of sequences associated with that group divided by the total number of sequences per sample.

3. RESULTS

3.1. Exploring CH4 sources in a BES-fed membrane-less MEC
A 1.3L MEC was inoculated using an anode from an operating MFC and an abiotic cathode. The MEC was inoculated and operated for 4 months with a BES concentration of 50 mM, enough to avoid methanogenesis according to common practices [19]. In spite of this, CH4 was produced. According to previous studies [8], methane production could be due to the activity of methanogenic Archaea, attached either to the anode, to the cathode or suspended in the cell medium (including those attached in the cell walls). A simple experiment was conducted in open circuit as a first assessment of the CH4 source (Scheme 1). The operating anode and cathode were separated into two different cells without the complementary electrode. A new abiotic cathode was added to the operating anode and a new abiotic anode was added to the operating cathode. Two different batch experiments were carried out in each cell: i) acetate as both electron donor and acceptor and ii) H2 as sole electron donor and CO2 as electron acceptor. CH4 was analyzed after 24 hours in the four experiments. The cell containing the operating cathode produced a low amount of CH4 with acetate. On contrast, higher CH4 production was observed when H2 and bicarbonate were the substrates, indicating that the most probable source of CH4 were the hydrogenotrophic methanogens growing on the cathode. The cell with the operating anode did not produce CH4, neither with H2 nor with acetate, showing the probable absence of acetoclastic methanogens and hydrogenotrophic methanogens in the anode.

“HERE Scheme 1”

3.2. Characterization of the MEC microbial community through qPCR
In order to sustain the experimental observations explained before, microbial characterization of the biomass was carried out by qPCR. For this, standard curves for qPCR were obtained of two plasmids containing 16SrDNA specific fragments cloned. In the case of Methanobacteriales and Archaea, a “mixed” fragment containing the two specific 16SrDNA regions described by Yu et al. [21] for these microorganisms was cloned in order to reduce the margin of experimental error and simplify the experimental procedure. Finally, three standard curves were obtained with specific primers (Figure 1) with a linear range of
detection spanning six orders of magnitude from $10^2$ to $10^7$ copies per reaction mix. A reproducible detection limit of 100 target copies was established in all the cases.

“HERE Figure 1”

Figure 2 shows the qPCR results pointing Geobacter, Methanobacteriales and total Archaea in the three DNA samples extracted from the MEC amended with 50 mM BES: anode, cathode and medium. Geobacter was predominantly present in the anodic biofilm ($4.6 \times 10^7$ gene copies·mg$^{-1}$) compared with total Archaea and Methanobacteriales order ($1.4 \times 10^5$ and $1.0 \times 10^5$ gene copies·mg$^{-1}$, respectively). On the other hand, the Archaea community was dominant in the cathodic biofilm ($1.7 \times 10^7$ gene copies·mg$^{-1}$) with the hydrogenotrophic Methanobacteriales as the predominant order ($1.5 \times 10^7$ gene copies·mg$^{-1}$), while the presence of Geobacter was much lower ($3.5 \times 10^5$ gene copies·mg$^{-1}$). With respect to MEC medium, no significant differences were encountered among the microorganisms studied with a presence of approximately $5 \times 10^5$ gene copies·mg$^{-1}$ (Figure 2).

“HERE Figure 2”

3.3. Pyrosequencing results
Anodic and cathodic DNA samples extracted from the MEC amended with 50 mM of BES were analyzed by high-throughput 16S rRNA gene pyrosequencing. Similar Archaea population was found in both anodic and cathodic biofilms, most of them belonged to Methanobrevibacter genus (around 98% in both cases) (Figure 3). With respect to bacterial population, noteworthy differences were detected between anodic and cathodic biofilms. In this sense, the dominant population in the anode was the exoelectrogenic bacterium genus Geobacter (72% of the total reads) (Figure 3). Other bacterium identified in this sample was Proteiniphilum (2%). A 12% of the population was designed as unclassified bacteria, since it was not possible to assign the sequences to any specific phyla. On the other hand, regarding the cathodic biofilm, the most represented genus was the homoacetogenic Acetobacterium (56%). Almost 34% of the sequences could not be ascribed to any particular phyla. In both samples, a 1-2% of the sequences were included in the “others” category as its representation was below 1% of the total reads.

“HERE Figure 3”

3.4. Scanning Electron Microscopy observations
Anode and cathode were sampled at the end of the period with 50 mM of BES and observed using SEM to characterize the biofilm structure and morphology (Figure 4). Graphite fibers of the anode were covered with an extracellular polymeric substance (EPS) while clear bacterial shapes of similar morphology were detected in the breaking points of the EPS structure. On the other hand, morphologically diverse bacteria (coccus and rods of different size) were observed on carbon cloth of the cathode, which showed a biofilm lacking in EPS with lesser biomass than the anode.

“HERE Figure 4”

3.5. Methanogenesis at different BES concentrations
As methanogenesis detection at 50 mM of BES concentration (50 mM) was surprising, this process was monitored in additional experiments using higher BES concentrations (100 and 200 mM). Current density and acetate concentration were monitored in each batch test (Figure
Cycle length increased from 160 h to 310 h when increasing BES concentration from 50 mM to 100 mM. When BES was further increased to 200 mM, the cycle length was extended and no decrease of current density was detected during the 450 h period that the cycle was operated. The decrease of current density observed for 50 mM and 100 mM experiments was linked to a decrease in acetate concentration. In spite of the current density observed for the experiment at 200 mM BES, very low decrease in acetate concentration was observed, as only 18 % of the initial acetate was consumed during the 450 h period. The fast increase in current density observed for example at time 120 h or 145 h in the 50 mM batch was due to the low acetate concentration linked to the manual agitation before medium sampling for acetate measurements.

"HERE Figure 5"

Gas composition was monitored at each BES concentration during 160 h (Figure 6). H₂ production was initially detected, reaching a peak in the first 20 hours of the cycle for each concentration step. During this first period, the rate of bioelectrochemical H₂ production was higher than that of hydrogenotrophic methanogenesis. A small amount of CH₄ was detected after the first 20 hours of batch-cycles, but it increased according to H₂ consumption, which was finally totally consumed and for this reason no H₂ was detected at the end of the cycles.

"HERE Figure 6"

Table 2 shows the CH₄ relative composition (Equation 3) over the time for the three cycles. With 50, 100 and 200 mM of BES concentration, the gas collected at the end of the cycles was mainly CH₄. The cycle with the initial concentration of 200 mM of BES was stopped after 450 h, but only a 23.5% decrease in acetate was observed (Figure 5). Homoacetogenic bacteria recycled H₂ to produce acetate which was in turn consumed by exoelectrogenic bacteria, generating an extremely long cycle. Acetate consumption rate in this experiment was 6.4 mg·L⁻¹·d⁻¹ during the first 450 h. This would represent a cycle length of more than 112 days if this rate was extrapolated until complete acetate consumption.

"HERE Table 2"

Table 3 summarizes the coulombic efficiency calculated for the three experimental periods. The values were higher than 100%, indicating the presence of H₂ recycling (further discussed below), which has been already reported for this type of systems [19]. Cathodic recovery (i.e. hydrogen recovered versus electrons arriving at the cathode) was almost null because the entire hydrogen production was being either recycled or used for methanogenesis. Moreover, around 25 % of the hydrogen produced would be used for methanogenesis applying simple electron balances as in Ruiz et al. [20].

"HERE Table 3"

Finally, samples from the cathodic biofilm were collected at the end of each cycle with different concentration of BES and the presence of order Methanobacteriales was analyzed by qPCR. A slight decrease (less than 1 log) of this Archaea was observed at concentrations higher than 50 mM of BES but no differences were appreciable among 100 and 200 mM.

4. DISCUSSION
This study reports CH₄ production in an MEC cell which was subjected to high BES concentration (50 mM) during a long-term period (4 months). Methanogenic activity under that BES concentration was surprising and, hence, it was investigated using two higher BES concentrations, 100 and 200 mM, for around 15 days each one. Figure 5 and 6 display the current density, acetate concentration and CH₄ and H₂ monitored for the whole cycles. To the best of our knowledge, this is the first MEC study with such high concentrations of BES. The high and increasing coulombic efficiency (being 194 % at 50 mM BES concentration and reaching 245% at 100 mM), suggested an important activity of homoacetogenic bacteria and H₂ recycling occurrence. In the cycle with 200 mM BES, methanogenesis was more inhibited as less methane was produced, but an important increase of homoacetogenic bacteria activity was observed, which is reflected in the extremely high CE of 1242% calculated for this experiment. Homoacetogenic activity was previously reported in membrane-less MEC [18,32], but our results demonstrate that the increase of BES concentration highly increments this issue: it results in a decrease of MEC performance because of H₂ recycling to acetate. This is corroborated with the increase of cycle length with 100 (310 h) and 200 mM BES (stopped at 450 h) (Figure 5). Despite this increase of homoacetogenic activity at high BES concentrations, qPCR showed a similar concentration of Methanobacteriales, perhaps, due to the inability of qPCR to discern DNA from active or inhibited microorganism. Longer operational periods with high BES concentration would be probably needed to observe a decrease in methanogens concentration.

The qualitative results shown in Scheme 1 indicated that CH₄ production was most likely due to hydrogenotrophic methanogens attached to the cathode, in accordance to Sasaki et al. [33]. Both the qPCR and pyrosequencing results with 50 mM of BES concentration (Figures 2 and 3) confirmed this fact: the Archaea population of the cathodic biofilm was dominated by the hydrogen-oxidizing genus Methanobrevibacter, of the Methanobacteriales order, which represented almost 98% of the total Archaea. The same values were detected in the Archaea of the anodic biofilm, where only 2% belonged to Methanoseta (an acetoclastic methanogen). The lack of acetoclastic methanogens in single chamber MEC was already reported in Lee et al. [34] or Parameswaran et al. [18] even when BES was not added. They argued that acetoclastic methanogens have lower affinity for acetate when compared to acetate-oxidizing ARB (KS = 177–427 vs 0.64 mg COD-L⁻¹, respectively [35,36]), thereby enabling the anode as electron sink rather than methanogenesis in acetate limited environments. The MEC operation in batch mode with 252 mg COD-L⁻¹ of initial acetate led to substantial acetate limitation in most of the reaction period and hence, the competition for acetate between ARB and methanogens seemed to be the key for the acetoclastic washout rather than different BES inhibition effect on H₂ - vs. acetate-oxidizing methanogens. However, qPCR results (Figure 2) show that the presence of Archaea in the anode was two orders of magnitude lower than in the cathode, and hence its activity in the anode was negligible when compared to the abundance of Geobacter, the most common exoelectrogenic microorganism found in acetate-fed bioelectrochemical systems [37]. The genera of Geobacteraceae family, like Geobacter, are able to oxidize acetate, colonize the electrode surface and conserve energy to support growth from electron transport to the electrode, using it as the sole electron acceptor. This gives them a competitive advantage over other microorganism in bioelectrochemical systems as MFCs and MECs [38].

On the other hand, the dominant bacterial genera identified in the cathodic biofilm was Acetobacterium (a homoacetogenic genus), able to oxidize H₂ and reducing CO₂ to produce acetic acid [39]. However, according to the presented pyrosequencing results, the ratio of Methanobrevibacter sp. with respect to Acetobacterium sp. in the cathode is ca 5:1. Acetobacterium was already isolated in MECs systems in previous studies [19,32]. Their presence in the cathodic biofilm, in addition to hydrogenotrophic methanogens, can be
explained by the continuous \( \text{H}_2 \) production in the cathode and because of its high concentration in the medium during the first hours of the cycle.

Once methanogens were detected, their presence in a cell with such high BES concentrations should be examined. Three different hypotheses could be put forward: i) biofilm mass transfer resistance; ii) BES degradation, and iii) microbial adaptation to high BES concentrations [40]. One hypothesis could be the mass transfer resistance of the EPS matrix of the biofilm. When this matrix is very thick, among other functions, it can hinder access and diffusion of chemical compounds (like antimicrobial agents) to the bacterial cells [41] leading to partial BES penetration and hence producing biofilm stratification with methanogens in the inner side. This would explain common BES observations: i) inhibition being effective in immature biofilms (i.e. short-term exposure), and ii) difficulty in reducing methanogens concentration when they are dominant in the system. Nevertheless, thanks to the SEM images, this hypothesis seems improbable since the cathodic biofilm looks very thin and apparently lacking of EPS.

Another hypothesis could be that the long-term exposure to BES resulted in the growth of microorganisms capable of BES degradation [40]. According to the pyrosequencing results, this hypothesis also seems improbable since any microorganism capable to either reduce or oxidize the sulfonate group of BES was detected in a high proportion. BES concentration was measured in several batch experiments following the methodology proposed by Rago et al. (submitted) and no significant changes were observed (data not shown), rebutting the hypothesis of its degradation. Moreover, Rago et al. (submitted) shows that biological BES degradation is a process that only occurs under aerobic conditions (as, for example, in the cathode of an MFC) and hence it is not expected under the anaerobic operation of an MEC. The last hypothesis could be that the long-term exposure to BES of the biofilm led to the development of some resistance to BES. This resistance has already been reported [42,43] and would be based on an inability to transport BES into the cell. As a structural analog of CoM, BES is a specific inhibitor of the terminal step of \( \text{CH}_4 \) biosynthesis and some methanogens are non-CoM dependent because they can synthesize CoM [44] and as such they can develop BES resistance [40]. Accordingly, development of BES adaptation could explain the experimental results obtained. The causes of this potential adaptation deserve further studies, but the operational conditions of our system (i.e. long cycles, high BES concentration and high cathodic hydrogen retention time) could be plausible reasons behind these results.

5. CONCLUSIONS

Despite the high concentration of BES used in a single chamber MEC, methanogenesis was not totally inhibited. The reason of this was likely due to the capacity of some Archaea (hydrogen-oxidizing genus \textit{Methanobrevibacter}, of the \textit{Methanobacteriales} order) to resist high BES concentration up to 200 mM. These results demonstrate that \( \text{H}_2 \) production in single chamber MEC is seriously challenged by \( \text{CH}_4 \) production even when operating at 100 mM BES concentration. Moreover, at higher BES concentration (200 mM), methanogenesis activity decreased resulting in an increase of \( \text{H}_2 \) recycling by homoacetogenesis. High presence of homoacetogenic bacteria undermines the efficiency of MEC to \( \text{H}_2 \) production. Moreover, further studies will be needed to prevent homoacetogenic bacteria from undermining methane production in MECs designed to favor hydrogenotrophic methanogenesis.

6. ACKNOWLEDGEMENTS

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Consolidat de la Generalitat de Catalunya, 2014 SGR 572). Laura Rago and Yolanda Ruiz are grateful for the grant received from the Spanish government (FPI and FPU, respectively).
REFERENCES


VITAE

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Yolanda Ruiz is a chemical engineer and PhD (2015) from the Department of Chemical Engineering at Universitat Autònoma de Barcelona. She is a member of the GENOCOV research group and she is conducting research on reducing the applied voltage requirements for hydrogen production in microbial electrolysis cells in view of the scaling-up of this technology.

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Figure captions

Scheme 1. Qualitative experiment to demonstrate CH₄ production in the cathode. The operating anode and cathode were separated into two different cells with abiotic complementary electrodes fed with either acetate (Subfigures 1a and 2a) or H₂ and CO₂ (Subfigures 1b and 2b).

Figure 1. Standard curves for 16SrDNA. The standards curves were constructed by using linear plasmid DNA and amplifying the corresponding target of -○- Geobacter, -●- Methanobacterales and -▼- Archaea. Values represent means (n=3) ± standard deviation.

Figure 2. Quantitative real-time PCR (qPCR) results for the cycle with 50 mM of 2-bromoethanesulfonate (BES) concentration. qPCR distribution of Geobacter (black), total Archaea (dark grey) and Methanobacterales (light grey) order on anode, cathode and medium in Microbial electrolysis cell (mean of triplicate values ± standard deviation).


Figure 4. Scanning Electron Microscopy (SEM) images of anodes and cathodes. (a, b) abiotic anodic graphite fiber; (c, d) abiotic carbon cloth covered with carbon black and platinum; (e, f) anodic biofilm; (g, h) cathodic biofilm. White squares represent the zones that are magnified in images b, d, f, and h.

Figure 5. Current density (mA · m⁻²) and acetate concentration (g/L). Experimental current density (solid line) and acetate concentration (---●--) profiles from periods with different 2-bromoethanesulfonate (BES) concentration: 50 mM, 100 mM and 200 mM.

Figure 6. Gas composition. Experimental CH₄ -○- and H₂ -●- profiles from periods with different 2-bromoethanesulfonate (BES) concentration: 50 mM, 100 mM and 200 mM.
FIGURES

Scheme 1.
Figure 1.

![Graph showing the relationship between log 16S rDNA gene copies and number of cycle/Cq.](image-url)
Figure 2.

- Anode
- Cathode
- Medium

16SrDNA copies · mg$^{-1}$

- $10^1$
- $10^2$
- $10^3$
- $10^4$
- $10^5$
- $10^6$
- $10^7$
- $10^8$
Figure 3.

A

Anode Archaea

Methanobrevibacter sp. 98%

Methanosaeta sp. 2%

B

Cathode Archaea

Methanobrevibacter sp. 97%

Unclassified Archaea 3%
**Anode Bacteria**

- **Geobacter sp.** 72%
- **Proteiniphilum sp.** 2%
- **Treponema sp.** 1%
- **Geoalkalibacter sp.** 1%
- **Others** 1%
- **Unclassified Bacteria** 12%
- **Unclassified Family** 9%
- **Unclassified Genera** 2%
- **Unclassified Desulfuromonadaceae** 1%

**Cathode Bacteria**

- **Acetobacterium sp.** 56%
- **Unclassified Clostridiales Inc. Sedis XI** 2%
- **Unclassified Genera** 2%
- **Others** 2%
- **Unclassified Family** 5%
- **Unclassified Bacteria** 34%
- **Dethiosulfitibacter sp.** 1%
Figure 4.
Figure 5.
Figure 6.
Laura Rago

Yolanda Ruiz

Juan Antonio Baeza

Albert Guisasola
Table 1. Primers and probes used for quantitative real-time PCR (qPCR).

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer/probes set</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Archaea</strong></td>
<td>ARC787F</td>
<td>ATTAGATACCCSBGTAGTCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ARC1059R</td>
<td>GCCATGCACCWCCTCT</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>ARC915 probe</td>
<td>AGGAATTGGCGGGGAGCAC</td>
<td></td>
</tr>
<tr>
<td><strong>Methanobacteriales</strong></td>
<td>MBT857F</td>
<td>CGWAGGGAAGCTGTAAAGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MBT1196R</td>
<td>TACCGTCGTCATCCTT</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>MBT929 probe</td>
<td>AGCACCACACCGTGGA</td>
<td></td>
</tr>
<tr>
<td><strong>Geobacter</strong></td>
<td>GEO561F</td>
<td>GCCATGCACCWCCTCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEO825R</td>
<td>GCGTGTAGCGGTCTTAA</td>
<td>[22,23]</td>
</tr>
<tr>
<td></td>
<td>Gbc1 probe</td>
<td>AGCACCACAAACCGTGGA</td>
<td></td>
</tr>
</tbody>
</table>

F: Forward primer; R: Reverse primer
Table 2. Methane relative composition ($\text{CH}_4 / (\text{CH}_4 + \text{H}_2)$). Gas composition in three cycles using different 2-bromoethanesulfonate (BES) concentration was analyzed after 20, 120 and 160 h.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>$\text{CH}_4 / (\text{CH}_4 + \text{H}_2)$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 mM BES</td>
</tr>
<tr>
<td>20</td>
<td>4.5</td>
</tr>
<tr>
<td>120</td>
<td>99.9</td>
</tr>
<tr>
<td>160</td>
<td>100</td>
</tr>
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</table>
Table 3. Coulombic efficiency (CE). Experimental CE obtained in microbial electrolysis cells using different 2-bromoethanesulfonate (BES) concentration.

<table>
<thead>
<tr>
<th>BES concentration</th>
<th>Coulombic Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM</td>
<td>194</td>
</tr>
<tr>
<td>100 mM</td>
<td>245</td>
</tr>
<tr>
<td>200 mM</td>
<td>1242</td>
</tr>
</tbody>
</table>
Highlights

- CH₄ was produced in a single-chamber MEC despite using 2-bromoethanesulfonate (BES)
- Methanogenesis was not inhibited despite 100 mM of BES in the media
- H₂-oxidizing Methanobrevibacter sp. represented 97% of total Archaea in the cathode
- Homoacetogenesis increased when methanogenesis decreased at 200mM of BES
- Development of BES adaptation could explain the experimental data