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Long-term continuous production of H$_2$ in a microbial electrolysis cell (MEC) treating saline wastewater

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ABSTRACT

A biofilm-based 4 L two chamber microbial electrolysis cell (MEC) was continuously fed with acetate under saline conditions (35 g/L NaCl) for more than 100 days. The MEC produced a biogas highly enriched in H$_2$ (>90%). Both current (10.6 ± 0.2 A/m$^2$ Anode or 199.1 ± 4.0 A/m$^2$ MEC) and H$_2$ production (201.1 ± 7.5 LH$_2$/m$^2$/d or 0.9 ± 0.0 m$^3$H$_2$/m$^2$/d) rates were highly significant when considering the saline operating conditions. A microbial analysis revealed an important enrichment in the anodic biofilm with five main bacterial groups: 44% Proteobacteria, 32% Bacteroidetes, 18% Firmicutes and 5% Spirochaetes and 1% Actinobacteria. Of special interest is the emergence within the Proteobacteria phylum of the recently described halophilic anode-respiring bacteria Geoalkalibacter (unk. species), with a relative abundance up to 14%. These results provide for the first time a noteworthy alternative for the treatment of saline effluents and continuous production of H$_2$.

Keywords:  
Biogas  
Biohydrogen  
Microbial electrolysis cell  
Saline wastewater  
Scanning electron microscopy  
High-throughput sequencing  
Geoalkalibacter

1. Introduction

A microbial electrolysis cell (MEC) is a well established microbial electrochemical technology (MET) used to produce high amounts of H$_2$ (Logan et al., 2008). A MEC profits from the activity of anode-respiring bacteria (ARB) embedded in anodic biofilms to break down the organic matter and harvest electrons that are chemically used at the abiotic chemical cathode to produce H$_2$. Nevertheless, the use of a biotic cathode is also possible as recently reported by Batlle-Vilanova et al. (2014) on the comparison of abiotic and biotic cathodes for microbial electrolysis of H$_2$. Usually, most of the MEC tests are performed using fresh wastewaters (i.e.: non saline) as anodic solutions that allow the successful development of electroactive biofilms from domestic wastewater (WW). Consequently, the application of such developed biofilms in MECs is mainly limited to the treatment of domestic WW. A successful treatment of other types of effluents such as saline WW which are significantly produced by the seafood, petroleum and leather industry will require the development of specific biofilms capable to work under such halophilic conditions. The enrichment of moderate halophilic biofilms composed of ARB could provide an alternative for the treatment of saline WWs which represent 5% of the WW generated worldwide and for which their treatment is usually limited by technical or economic constraints (Lefebvre and Moletta, 2006). However, another issue that emerges from an extensive overview of the literature on MECs (Logan et al., 2008) is the lack of studies working under saline conditions. Moreover, real WW treatment will require continuously operated MECs that will make necessary the development of a well attached-mature biofilm as an essential prerequisite for long-term operation of ARB biofilm-based METs.

Although the scalability of the MEC technology is of the main interest within the MET-related scientific community only a few attempts have been done to bring the MEC technology from the bench-via the pilot-to the full-scale size. In this context it is worth mentioning the works by Escapa et al. (2015), Gil-Carrera et al. (2013), Heidrich et al. (2013, 2014) who have very recently done significant attempts to scale-up the technology by operating 100 L size MECs fed with real domestic WW. Independently of the drawbacks encountered by these two engineering research groups, they have shown for the first time that the MEC technology is finally capable of converting low strength domestic WW to produce
a gas stream highly enriched in hydrogen (~100%) and most importantly at very similar operational conditions as the ones of conventional WW treatment plants. The aim of the present work was to operate a stable biofilm-based MEC in continuous mode for more than 100 days under saline conditions. A mature and moderate halophilic anodic ARB biofilm was first enriched from saline anoxic sediments under potentiostatic control during three acetate fed-batch cycles and then, operated under continuous mode. The long-term operation corroborated not only that a mature biofilm had well developed on the anode but also it confirmed the overall stability of the system. At the end of the experiment, the anode was removed and 1) the microbial composition by 16S rDNA sequencing and 2) biofilm attachment by scanning electron microscopy were analyzed. To the best of the authors’ knowledge, this is the first work reporting such a long-term performance of a continuously operated MEC under saline conditions.

2. Materials and methods

2.1. General conditions

All chemicals were of analytical or biochemical grade and were purchased from Sigma-Aldrich and Merck. If not stated otherwise, all potentials provided in this manuscript refer to the SCE reference electrode (KCl 3.0 M, +240 mV vs. SHE). Materials, Mates, La Guillétière 38700 Sarceins, France). All media preparations were adjusted to pH 7.0. Bioelectrochemical experiments were conducted under potentiostatic control and incubations were performed at 37.0 °C.

2.2. Metabolite and biogas analysis

The model substrate was acetate and its concentration was determined by liquid injection into a gas chromatograph (Clarus 580 GC, PerkinElmer). Biogas composition (CH₄, CO₂, H₂ and N₂) was determined using a gas chromatograph (Clarus 580, PerkinElmer) coupled to thermal catharometer detector, as described elsewhere (Quéméneur et al., 2012). The biogas was periodically analyzed for its H₂S content using a second gas chromatograph (Clarus 480, Perkin Elmer) equipped with an RTUBond-SiOH column and a molecular sieve (RTMolsieve). Operating conditions were as follows: the carrier gas was helium at a pressure of 250.0 kPa under a flow rate of 4.0 mL min⁻¹; temperature of the injector and the detector was fixed at 150.0 °C. Throughout the manuscript the average gas composition is reported. During Phase II the gas was daily sampled. During Phase III the gas was sampled before increasing/changing the substrate concentration. Averages and standard deviations are calculated taking into account two replicates. The model substrate used here was acetate, real saline WW is characterized for its high content of organic matter (Lefebvre and Moletta, 2006). The treatment of such WW in a MEC might follow conventional degradation steps: hydrolysis, acidogenesis, acetogenesis and finally, electron harvesting by halophilic ARB. During fed-batch chronoaeropermetric biofilm growth, the medium was supplemented with 50.0 mM of 2-(N-morpholino)ethanesulfonic acid (MES) to buffer the pH at around 7.0. For continuous MEC operation the medium inlet was adjusted at pH 7.0 with 1 M NaOH. Either during fed-batch or continuous operation, pH of the effluent slightly increased and remained relatively neutral (7.4 ± 0.2).

2.5. Bench scale 4L microbial electrolysis cell (MEC) configuration

A scheme of the unstirred up-flow microbial electrolysis cell (MEC) is presented in Fig. 1. The MEC body was a polycarbonate cylinder constituted of several parts (from outside to inside): (1) a water jacket (20.0 cm diameter and 30.0 cm height) used to keep a constant temperature of 37.0 °C (see cross-section and top view in Fig. 1); (2) the MEC body (16.0 cm diameter and 30.0 cm height) that contained both anode and cathode, had a total volume of 6.0 L and a final working volume of 4.0 L (i.e., after placing electrodes). As cathode material, a flexible 1 cm thick piece of graphite felt (12.0 cm diameter and 20.0 cm height) with a projected surface area of 754.0 cm² (grade RVG 4000, Mersen, France) was used. The graphite felt was slightly compressed and cylinder-shaped by a welded grid of titanium as electron collector, with two wires piercing the upper part of the MEC body to allow the connection with the potentiostat. An anionic exchange membrane (AEM) was placed between anode and cathode, as shown in Fig. S1 (FIA-PK, FuMA-Tech GmbH, Germany). The presence of a membrane in a MEC avoids H₂ recycling at the anode. Furthermore, the use of a AEM usually leads to a better electrochemical performance in comparison to other ion exchange membranes (Rozendal et al., 2008). As cathode material (188.0 cm² of catholyte exposed surface area), an empty cylindrical stainless steel 254SMO tube with an external diameter of 2.1 cm was located at the center of the MEC (Flowell, France). Through the cathode, a 2.0 mm diameter hole was drilled at the top of the electrode to collect the biogas. The volume of biogas was daily measured by displacement of water in a graduated column. Additionally, a SCE reference electrode was used to monitor a constant applied potential of +200.0 mV (+444.0 mV vs. SHE) at the anode.

2.6. MEC start-up and operation: phase I, II and III

At all times the anode potential was constantly fixed at +200.0 mV vs. SHE (+444.0 mV vs. SHE) instead of adding a specific voltage to the MEC circuit (see please Appendix). During
Phase I, the MEC was initially operated in fed-batch mode for at least three chronoamperometric cycles to grow an anodic biofilm (Fig. 2). By using a VSP Potentiostat/Galvanostat interfaced to a VMP3B-80 Current Booster unit (BioLogic Science Instruments, France) it was possible to guarantee an homogenous distribution of the potential along the anode electrode. To initiate the biofilm growth, the MEC was first inoculated with 10.0% w/v of saline sediments and then fed with modified Starkey medium supplemented with a progressive increase of COD concentration as fed acetate: 0.64, 0.96 and 1.28 g/L (Fig. 2). Before every fed-batch cycle, the medium was vigorously flushed with N₂ gas (purity ≥ 99.9999, Linde France S.A.) to provide anaerobic conditions for at least 30.0 min using a commercial air stone (or aquarium bubbler). During these fed-batch cycles, current production was monitored as a direct signal of the development of the electroactive biofilm on the anode. During Phase II and after chronoamperometric proof of the presence of a mature biofilm on the electrode (Fig. 2), the MEC was operated in continuous mode at a flow rate of 7.5 L d⁻¹, corresponding to an HRT of about 12.0 h. For continuous MEC operation, no special precautions were taken to remove oxygen from the medium and no sediments were added to the medium composition. Finally, during Phase III, an increase in COD concentration as fed acetate from 0.64 to 6.42 g/L was tested to evaluate whether the MEC could improve its overall performance.

2.7. DNA extraction and MiSeq sequencing of anodic biofilm samples

At the end of the long-term continuous operation (after 100.0 days), the MEC was opened and the anodic biofilm was vertically sampled at three different locations (from bottom to top: at 6.0, 12.0 and 18.0 cm) to evaluate the homogeneity of the microbial population regardless of its location on the electrode material (see Fig. S2). Due to the homogenous distribution of the applied potential we obtained an uniform composition along the anode as indicated by the high similarity in CE-SSCP profiles (Fig. S2). The biomass was heat-treated and underwent bead-beating as done in Rochex et al. (2008) to facilitate DNA extraction and purification with the QIAamp DNA mini kit (QIAGEN, Courtaboeuf, France). DNA amount and purity in extracts was confirmed by spectrophotometry (Infinite NanoQuant M200, Tecan, Austria). Extracted DNA was stored at −20 °C until further use. The V3–4 region of the 16S rRNA gene was amplified with the forward primer CTTTCCCTACGACGCTCTTCCGATCTTACGGRAGGCAGCAG and the reverse primer GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAA TCCT plus the respective linkers over 30 amplification cycles at an annealing temperature of 65.0 °C. In a second PCR reactor of 12 cycles, an index sequence was added using the primers AATGATTACGGCGACACCTAGATCCTACAGCTCACACGAC and CAACAGCACGCACATACGAGAT-index-GTGACTGGAGTTCAGACGTGT. The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer’s instructions for sequencing of paired 250 bp reads. Sequencing-related work was done at the GeT PlaGe sequencing center of the genotoul life science network in Toulouse, France (get.genotoul.fr).

38100 forward and reverse sequences were retained after assembly and quality checking using an slightly modified version of the Standard Operation Procedure by Kozich et al. (2013) in Mothur version 1.33.0. SILVA release 102 as provided by Schloss et al. (2009) was used for alignment and as taxonomic outline. Using Mothur, representative sequences of deltaproteobacterial OTUs (at the 3.0% level) with a relative abundance of more than two percent were aligned with select deltaproteobacterial sequences (see Table S1) from the 16S rRNA-based phylogenetic tree harboring all type
strains of all species with validly published names up to June 2013 (The All-species Living Tree, release LTTPS115 [Yarza et al., 2008]). The software PAUP* (version 4.0b10) was used to infer a phylogeny using the criterion of maximum parsimony (Swofford, 1989). Bootstrap support was calculated using 1500 replications. SumTrees (version 3.3.1) of the DendroPy package (version 3.12.0) was used to map bootstrap values to the best phylogeny (Sukumaran and Holder, 2010). Sequences of most abundant OTUs found in the biofilm were deposited in the NCBI Genbank database under the following accession name: "PRJNA273803".

2.8. Biofilm analysis by scanning electron microscopy (SEM)

The anode was removed from the MEC and vertically sampled for SEM after 100 days of operation. Graphite felt electrode samples (0.5 × 0.5 cm) were carefully fixed in a 2.5% glutaraldehyde solution in a 0.1 M sodium cacodylate buffer (0.1 M, pH 7.4) overnight at 4 °C. After two serial wash steps in sodium cacodylate buffer (0.2 M), samples were dehydrated in a graded ethanol series, desiccated on a Leica EM CPD300 critical point dryer and then coated with Platinum on a Leica EM MED020 ion sputter. Finally, electrode samples were observed with a FEI Quanta 250 scanning electron microscope, with an accelerating voltage of 5.0 kV.

2.9. Calculations

The performance of the MEC was assessed in terms of geometric/volumetric current density and geometric/volumetric hydrogen production rate as follows. While the working volume of the MEC (ca. 4 L) was considered for both volumetric calculations (in m^3/m^2/mEC d^-1 and A/m^2_MEC), the projected surface area of the anode (754.0 cm^2) and cathode (188.0 cm^2) were used to calculate the geometric current density and geometric H_2 production rate (in A/m^2_Anodo and L H_2/m^2/Cathode d^-1), respectively. Coulombic efficiency was calculated in terms of electrons recovered from the substrate as follows (Logan et al., 2008): CE = (M_e / F) * (F / b_cat * q / dF). The number of moles of hydrogen was calculated as n_H2 = n_fl * n_cat for the MEC operated in continuous mode during Phase II and III (Batlle-Vilanova et al., 2014). The number of moles of hydrogen was calculated as n_H2 = (P_H2 / (R * T)), where P_H2 (L) is the volume of hydrogen measured, P (atm) is the atmospheric pressure measured in the laboratory, R (L atm)/(mol K)) is the ideal gas constant and T (K) is the temperature. The theoretical amount of hydrogen that could be produced from the measured current was calculated as n_fl = C_b / (2 * F), where C_b is the total coulombs calculated by integrating the current over time, Z is the moles of electrons per mole of hydrogen and F is the Faraday’s constant.

3. Results and discussion

3.1. Phase I: MEC start-up and development of a moderate halophilic anodic biofilm

In order to obtain a mature anodic biofilm, the up-flow MEC was operated in fed-batch mode for three cycles and the current production was monitored as sole indicator of the growth of the electroactive biofilm. The moderate halophilic conditions were ensured by feeding the MEC with saline (35.0 g/L NaCl) modified Starkey medium. The MEC was re-inoculated two more times with 10% of sediments at the beginning of each cycle. Fig. 2 shows the chronopotentiometric (CA) current production, i.e.: the electroactivity of the biofilm at increasing COD concentration as fed acetate ranging from 0.64 to 1.28 g/L. As shown, the biofilm started to grow after approximately 1 day of poising the anode at +200.0 mV vs. SCE (+444.0 mV vs. SHE). This was illustrated as an exponential-like current production trend, characteristic of the growth of anode respiring bacteria (ARB) able to produce significant sustained current densities (j > 1 A/m^2) and mature biofilms. Such growth is a major prerequisite for long-term continuous operation of a biofilm-based MET such as an MEC. In the first cycle, a short lag phase of 1 day was observed (Fig. 2). In the 2nd and 3rd fed-batch cycles, the current immediately started as a clear indicator of the presence of an efficient electroactive biofilm on the anode material (see microscopic analysis section).

3.2. Phase II: MEC continuous operation performance

After successful biofilm formation during three batch cycles (Fig. 2), the MEC was switched from batch to continuous feeding mode with an HRT of 12.0 h. The MEC performance of the long-term operation is presented in Fig. 3. Since no sediment was added to the medium all along the continuous feeding, the observed current was only resulting from the biofilm activity. Instead of adding a specific voltage to the MEC circuit, a potentiostat was used to constantly poise the anode potential at +200.0 mV vs. SCE (Nam et al., 2011). Consequently, the observed potential at the cathode slightly oscillated around ~1363.6 ± 165.5 mV during all continuous operation. Such electrochemical conditions were favorable to H_2 production at the cathode. The average gas composition at the cathode was close to 90% H_2, i.e. (in %): H_2 87.8 ± 15, CO_2 3.7 ± 0.3 and CH_4 2.0 ± 0.1. The low concentrations of O_2 (3.2 ± 0.6%) and N_2 (3.3 ± 0.9%) were very likely caused by the manual sampling procedure. The minor amounts of detected CH_4 were probably due to gas diffusion from the anodic chamber through the membrane. This result clearly points out that the acetate was efficiently converted at the anode to electrons that finally were converted into H_2 at the cathode. Moreover, the amount of biogas being produced at the anode was negligible in comparison to the gas produced at the cathode. Thus, no liquid displacement was observed in the column used to measure biogas. Nonetheless, to conduct a more detailed analysis of the composition of the gas atmosphere at the anodic chamber, gas samples were regularly analyzed. Methane was the main compound detected since no methanogenesis inhibitor was added, i.e., (in %): CH_4 68.1 ± 12.7, CO_2 18.1 ± 5.9, H_2 11.7 ± 5.7, O_2 0.3 ± 0.3, N_2 1.8 ± 1.4.

Once the continuous flow of medium was enabled (Fig. 3), the overall production gradually increased with a stable performance around days 30–35 with maximal current densities of 2.3 ± 0.0 A/m^2_Anodo or 43.5 ± 0.7 A/m^2_MEC and hydrogen production rates of 51.4 ± 1.9 L/m^2/mEC d^-1 or 0.2 ± 0.0 m^3/m^2/mEC d. Interestingly, the initial pH adjustment to neutrality was enough to keep a relatively stable neutral environment in the anodic chamber (pH: 7.4 ± 0.2). In MECs where an AEM is used the working principle of the membrane charge transport relies in the transport of (ideally) hydroxyl ions from cathode to anode (Rozendal et al., 2008). Some of the available hydroxyl ions produced due to the reduction of H_2O migrated to the anode chamber where they have slightly increased the anolyte’s pH. The stable neutral pH was probably due to the available hydroxyl ions produced due to the reduction of H_2O. The stable neutral pH was probably due to the available hydroxyl ions produced due to the reduction of H_2O. The stable neutral pH was probably due to the available hydroxyl ions produced due to the reduction of H_2O. The stable neutral pH was probably due to the available hydroxyl ions produced due to the reduction of H_2O.
caused by the incapability of negative charged ions like OH\(^-\) to sufficiently cross the membrane and then migrate to the anode chamber. A possible explanation for the loss of membrane performance might be the formation of biofouling which can block the available pores of the membrane (Erable et al., 2012).

The MEC-based process described here has been used to convert a readily substrate by ARB such acetate into H\(_2\) under saline conditions. However, to fully demonstrate that such technology could be considered as a novel alternative treatment of saline effluents, our MEC-based process should be tested with real saline wastewater. A work that has shown very promising results and that it is currently under development (Marone et al., 2014).

The coulombic efficiency (CE) of the system was evaluated as well as the cathodic hydrogen recovery (r\(_{\text{cat}}\)), a well-accepted parameter to quantify to what extent the measured H\(_2\) was actually recovered from the observed current.

In Phase II, the maximum CE and r\(_{\text{cat}}\) were found at day 14 of the continuous operation with a value close to 80.0% and 40.0% (day 14), respectively. This observation indicates that at day 14 i) at least 80.0% of the substrate was biologically recovered as electrons and ii) that 40.0% of those electrons were further converted into H2 at the cathode. Although the COD removal stabilized (from day 30) at 75% both r\(_{\text{cat}}\) and CE rapidly decreased. Thus, both r\(_{\text{cat}}\) and CE are not representative of the continuous operation during Phase II in which the MEC was operated under a single substrate concentration of 1.28 g/L COD in fed acetate. Such rapid decrease and stabilization of CE (~10%) and r\(_{\text{cat}}\) (close to zero) suggests that i) parallel metabolic reactions occurred as a possible electron sink (Batlle-Vilanova et al., 2014) and ii) there was an important loss of electrons probably due to detected but unquantifiable CH\(_4\) production (~70%) in the anode chamber.

### 3.3. Phase III: increasing acetate concentration test under continuous feeding

After day 54 (see Fig. 3), an increase in substrate concentration was tested to evaluate whether the MEC could improve its overall performance. Fig. 4 shows the current density and H\(_2\) production rates after 50 days of continuous operation of the MEC under increasing concentrations of acetate (see also Fig. S3). As expected, the performance of the MEC showed a linear increasing trend depending on the amount of substrate fed. The maxima average values of both current densities (10.6 ± 0.2 A/m\(^2\)Anode or 199.01 ± 4.0 A/m\(^3\)MEC) and H\(_2\) rates (201.1 ± 7.5 L/m\(^2\)Cathode.d or 0.9 ± 0.0 m\(^3\)H\(_2\)/m\(^2\)MEC.d) were obtained when the MEC was continuously operated with a COD concentration as fed acetate of 6.42 g/L. Usually the current density increases with the addition of higher substrate concentrations until saturation kinetics is observed. When considering that no saturation is reached, it might suggest that the MEC was capable to convert even higher concentrations of substrate.

During Phase III we observed a similar trend as the one observed in Phase II regarding COD removal, CE and r\(_{\text{cat}}\). Although an important fraction of the COD in fed acetate was removed (50–70%) the values obtained for CE (~20%) and r\(_{\text{cat}}\) (~20%) indicated again an undetected gas leakage in the system.

The relatively stable CE (Fig. 4), with the exception of the value at 0.64 g/L COD due to the difference in current density production (see Fig. S3), indicated that the MEC was able to convert the substrate into electrons with a stable but low efficiency of 20%. When considering the conversion of substrate into H\(_2\) (r\(_{\text{cat}}\)), it can be noticed that although the r\(_{\text{cat}}\) increased during Phase III, the highest value of r\(_{\text{cat}}\) was 24%.

Although r\(_{\text{cat}}\) possibly increased due to the lack of substrate limitation, r\(_{\text{cat}}\) is still considered lower than previously reported values in the literature. Possible reasons for this relatively low value of r\(_{\text{cat}}\) (<25%) during this test and after considering that the MEC was fed with synthetic medium, could be the followings: first, the architectural design of the MEC could have had an impact on the overall substrate conversion. The MEC was fed from the bottom of the system (Fig. 1) and the graphite felt anode was placed in a parallel orientation with respect to the direction of the flow. Thus, it is likely that at higher concentrations of substrate there was not proper mass transfer through the material. Second, it is probable that the graphite felt anode material suffer of biofouling due to its open pore structure that provided not only a habitat for the growth of ARB but also a trap for cellular material.

The findings presented here are in good agreement with previous and current literature in terms of current density (j), H\(_2\) production rate, Coulombic efficiency (CE) and cathodic H\(_2\) recovery (r\(_{\text{cat}}\)) (Kundu et al., 2013). However, the scarcity of works reporting the operation of bench scale liter MECs for the production of H\(_2\) under continuous flow conditions prohibits an objective comparison of results (with the few exceptions of pilot scale MECs, e.g.: (Cusick et al., 2011)). Nonetheless, the MEC performance is highly important, especially when considering the long-term
operation (>100 d) of the MEC operated under saline conditions. These results are even more remarkable when compared with optimized lab scale MECs that are specifically designed to, e.g.: i) substantially increase mass transfer, ii) reduce electrode spacing, iii) enhance the H₂ evolution reaction with highly catalytically active cathode materials or iv) improve the electrodes arrangement (Cheng and Logan, 2011; Jeremiasse et al., 2011; Liang et al., 2011).

3.4. Comparison of the obtained performance with highly scalable MECs

Only a few significant attempts have been done to scale-up the technology by operating 100 L size MECs fed with real domestic WW (Escapa et al., 2015; Gil-Carrera et al., 2013; Heidrich et al., 2013, 2014). When compared our results to the recently reported performance by scalable-MECs one can clearly notice that our MEC has outperformed those reported in Table S3. The main difference among these works and ours is the high conductivity used here (9 S/m) due to the addition of 35.0 g/L of NaCl in comparison to domestic WW that typically has a conductivity of only 1 S/m.

A possible explanation why our MEC outperformed those reported in Table S3 might be the constant experimental conditions used here, e.g.: (i) potentiostatically fixed anodic applied potential (+200 mV vs. SCE), (ii) constant temperature (37 °C), (iii) neutral pH conditions (7.0) and (iv) use of a synthetic medium containing a non fermentable substrate such as acetate.

Although current densities and H₂ production rates reported by Escapa et al. (2015), Gil-Carrera et al. (2013), Heidrich et al. (2013, 2014) are still considered low but promising (Table S3), these two engineering research groups have shown for the first time that the MEC technology is finally capable of converting real WW into a gas stream highly enriched in H₂ (~100%) and most importantly at very similar operational conditions as the ones of conventional WW treatment plants.

3.5. Structure and composition of the anodic bacterial community with an emphasis on the deltaproteobacteria

Using Illumina MiSeq technology, 38100 partial 16S ribosomal DNA gene sequences were obtained out of which 44% were assigned to the phylum Proteobacteria, 32% to Bacteroidetes, 18% to Firmicutes, 5% to Spirochaetes and 1% to Actinobacteria. An annotated abundance table is given in the Appendix as Table S1. Several microorganisms within the deltaproteobacteria class have been proved to possess electroactivity. These microorganisms repeatedly appear in anodic biofilm communities and in well performing microbial electrochemical technologies (METs) that produce current densities ≥ 1 A/m² (Yates et al., 2012). We selected one representative sequence from each of deltaproteobacterial operational taxonomic units (OTUs) with an abundance of more or equal to 2.0% for the inference of a phylogenetic tree (Fig. 5). The four OTUs in Fig. 5 make up more than 34% of all sequences.

OTU 1 (with an abundance of 14.1%) was closely related to the well-known anode-respiring bacterium (ARB) Geobalkalibacter subterraneus. G. subterraneus is capable of attaching to electrode materials under similar experimental conditions as the ones used here, i.e. saline medium, 37.0 °C, pH 7.0, constant applied potential.
among others (Carmona-Martínez et al., 2013). Based on its position within the phylogeny, OUT 1 very likely possesses electroactive properties.

The close relatedness between OTU 4 (8.2%) and Desulfuromonas acetoxidans suggests that OTU 4, as D. acetoxidans, may be an acetate-oxidizing ARB. D. acetoxidans has been previously detected in electroactive biofilms enriched from sediments and preliminary characterized as a pure culture (Bond et al., 2002). Similarly, it was inferred that OUT 10 possesses an electroactive function within the anodic biofilm community as an additional ARB enriched in this study due to the relatedness of OTU 10 (2.1%) to Desulfuromusa ferrireducens and Geosynchrobacter electrodiphilus, two psychrophilic dissimilatory iron and anode respiring bacteria (Holmes et al., 2004; Vandieken et al., 2006). The fact that several ARB belonging to the Deltaproteobacteria class coexisted within the biofilm could have been facilitated by the three dimensional geometry of the graphite felt anode. Its open pore structure may be a trap for cellular material and thus provide spatially separated habitats supporting growth of several ARB.

The inference of electroactivity to OTU 3 (10.0%) was not straightforward from its position in the phylogeny. Instead, organisms grouped into OTU 3 might be sulfate-reducing bacteria as the sequences are phylogenetically closely related to Desulfovibrio halophilus (Caumette et al., 1991) and Desulfovibrio marinus (Ben Dhia Thabet et al., 2007). OTU 3 may be able to oxidize \( \text{H}_2 \) and/or acetate and use sulfate as terminal electron acceptor under moderate halophilic conditions. However, this reaction has unlikely happened as only traces of \( \text{H}_2 \text{S} \) (i.e., \( \text{H}_2 \text{S} \leq 0.07 \pm 0.01 \% \)) were detected in the anodic chamber. Microbial reduction of the only sulfate source introduced to the reactor by traces in the oligo-elements solution could therefore not explain the quantity of OTU 3 sequences. Nonetheless, the initial 23 g of MES buffer added during Phase I of biofilm growth could have provided an additional source of sulfate for the sulfate-reducing bacteria to persist.

### 3.6. Qualitative imaging of biofilm deposition on the anode

In order to evaluate the biofilm shape and deposition on the anodic surface, scanning electron microscopy (SEM) was used to conduct a qualitative inspection as described in the experimental section. SEM was chosen over confocal laser scanning microscopy (CLSM) due to the three dimensional arrangement of the electrode material which would prohibit a reliable quantitative analysis. Fig. 6 shows representative SEM pictures of multiple samples of the anodic biofilm developed after more than 100 days of continuous operation (see also Fig. S4). As expected, most of the carbon felt fibers were covered by amorphous layers. Although these layers did not resemble to those observed in pure cultures or highly enriched anodic biofilms in which at higher magnifications a characteristic bacterial shape can be observed, the layers do indicate the presence of a microbial biofilm attached to the electrode material.

In the case of pure culture or highly enriched biofilms derived from domestic wastewaters, the bacterial cells usually accumulate as densely packed amorphous layers that completely cover the electrode material. In the present work, saline sediments were used as inoculum source and the medium contained an important amount of NaCl (35.0 g/L). Therefore, the amorphous aggregates observed in Figs. 6 and Fig. S4 could be caused not only by a microbial biofilm but also by the organic matter contained in the sediments and/or salt precipitates possibly formed due to the long-term operation. Such deposit could have decreased the total available surface area of the electrode. Nonetheless, when closely analyzing the pictures with the 10 μm scale bar in Figs. 6 and S4, several rod shaped structures can be seen. Such characteristic shape structures resemble those of the main bacteria detected in the microbial analysis of the anodic biofilms. At the 10 μm scale bar pictures, some kind of web-like extracellular material can be seen and can likely be attributed due to sample preparation as suggested by others (Badalamenti et al., 2013). To affirm whether this extracellular material could be classified as a microbial nanowire, further measurements are required to prove that these putative wires actually perform the so called “long-range electron transport” in a catalytically active microbial biofilm (Malvankar and Lovley, 2012).

### 4. Conclusions

The continuous operation of a MEC for more than 100 days and under saline conditions showed a significant \( \text{H}_2 \) production rate. A biofilm was first enriched in batch mode for three chronoamperometric cycles. The biogas stream was highly enriched in \( \text{H}_2 \) (>90%). When considering that the system was operated under saline conditions, both current and \( \text{H}_2 \) production rates were highly remarkable and promising for further application. Although the COD removal was important in the system, the electrons from COD were not found either as CE or \( r_{\text{CE}} \). Such lost of electrons evidenced parallel metabolic reactions and an important leak in the system. The microbiological analysis of the biofilm showed a significant selection with mainly Deltaproteobacteria colonizing the anode. This MEC-based process is a promising alternative to other technologies to treat saline wastewaters.

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**Fig. 6.** Exemplary scanning electron microscopy pictures of the anodic electroactive biofilm observed after more than 100 days of MEC continuous operation. Scale bars are indicated in the bottom right for all pictures.
samples used for plotting CE-SSCP profiles/pyrosequencing and C. Pouzet from the “Plateforme d’imagerie FR-AIB, TRI-Genotoul” for technical assistance with SEM.

References


