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Alessandro Carmona Martinez, Eric Trably, Kim Milferstedt, Rémy Lacroix, Luc Etcheverry, et al.. Long-term continuous production of H2 in a microbial electrolysis cell (MEC) treating saline wastewater. Water Research, 2015, 81, pp.149-156. 10.1016/j.watres.2015.05.041 . hal-01167881

HAL Id: hal-01167881
https://hal.science/hal-01167881v1

Submitted on 28 May 2020

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Appendix. Supplementary data for:

Long-term continuous production of H_2 in a microbial electrolysis cell (MEC) treating saline wastewater

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Keywords: Biohydrogen, Microbial electrolysis cell, Saline wastewater, Scanning electron microscopy, 454 Pyrosequencing, *Geoalkalibacter*

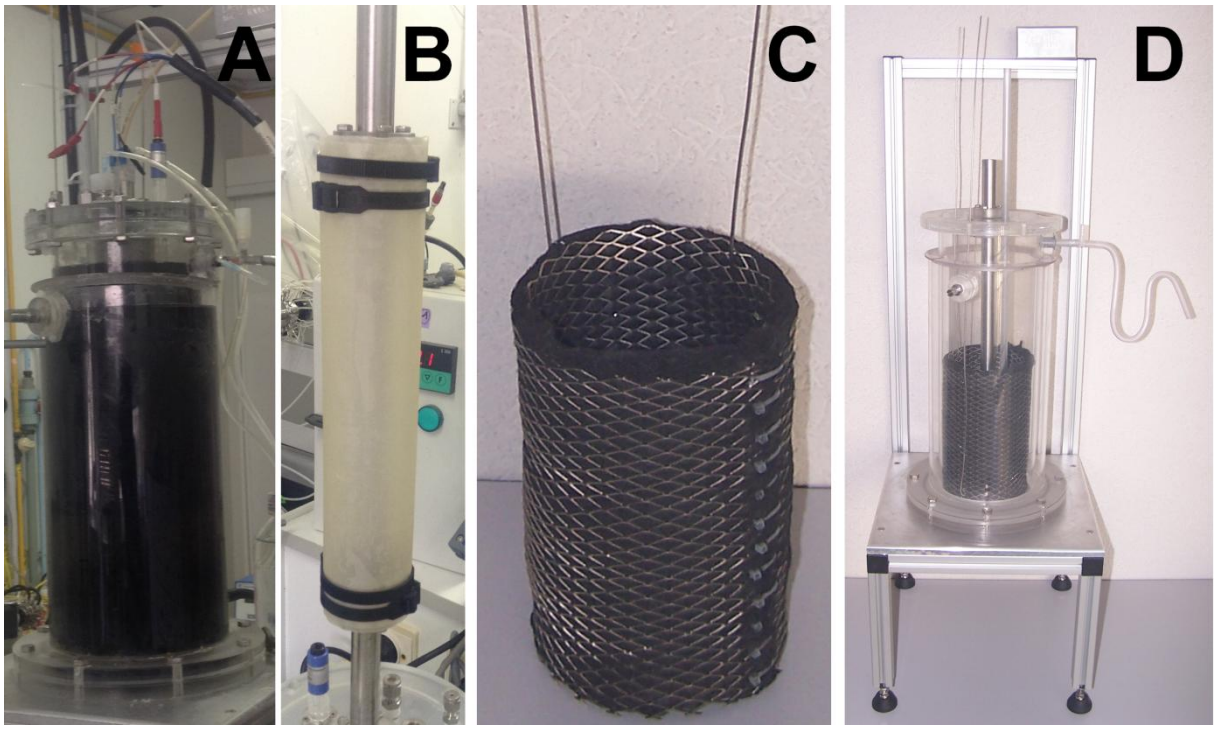

Fig. S1 - A) MEC in operation; B) Anionic membrane folded around an empty cylindrical stainless steel 254SMO tube cathode; C) Folded graphite felt as anode and D) Empty MEC designed and built by project partners at the Laboratoire de Génie Chimique, UMR 5503, Toulouse and 6T-MIC Ingénieries as described by (Rousseau et al. 2013).

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1. CE-SSCP fingerprinting of electroactive biofilm samples after 100 days of operation

A Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) method was used for PCR products fingerprinting. To perform CE-SSCP fingerprinting, one microliter of the appropriate dilution of PCR products was mixed with 18.925 µL of formamide and 0.075 µL of internal standard GeneScan ROX (Applied Biosystems). Samples were heat-denatured at 95°C for 5 min and re-cooled directly in ice for 5 min. CE-SSCP electrophoresis was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems) in 50 cm capillary tubes filled with 10% glycerol, conformation analysis polymer and corresponding buffer (Applied Biosystems). Samples were eluted at 12kV and 32°C for 30 min (see Fig. S2). CE-SSCP profiles were aligned with an internal standard, ROX, to consider the inter-sample electrophoretic variability. The CE-SSCP profiles (Fig. S2) were normalized using the Statfingerprints library (Michelland et al. 2009) in R software version 2.9.2 (R. Develpment Core Team 2010), with a standard procedure as described elsewhere (Fromin et al. 2002).

2. CE-SSCP profile description

Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP), a fingerprinting pattern analysis based on 16S rDNA sequences is a widely used technique considered as an "image" of the whole microbial diversity given by discrete peaks that emerge from the subpeak background (Loisel et al. 2006). Fig. S2 presents a representative 16S rRNA gene-based CE-SSCP fingerprint profile of multiple analyzed samples of the sediment used as inoculum before the MEC start-up (Fig. S2A) and CE-SSCP profiles from the enriched anodic electroactive biofilm collected at the end of Phase III (Fig. S2: B1-3). While, the CE-SSCP profile obtained from the sediment depicted a complex community (Fig. S2A), biofilm CE-SSCP profiles showed a slightly more defined structure regardless their location on the electrode material (Fig. S2: B1, B2 and B3). In particular, the well-defined prominent peak found at about 350 (arb. units) on the "x" axis clearly points out that a high microbial selection occurred likely due to the constant experimental conditions used during biofilm development (Phase I) and MEC continuous operation (Phase II and III): e.g., anode potentiostically controlled, pH, temperature and single substrate feeding. A finding well in agreement with other studies in which this type of high microbial selection in anodic biofilms has been mainly (and almost exclusively) observed in systems inoculated with domestic wastewater and fed with a single non-fermentable substrate such as acetate (Harnisch et al. 2011).

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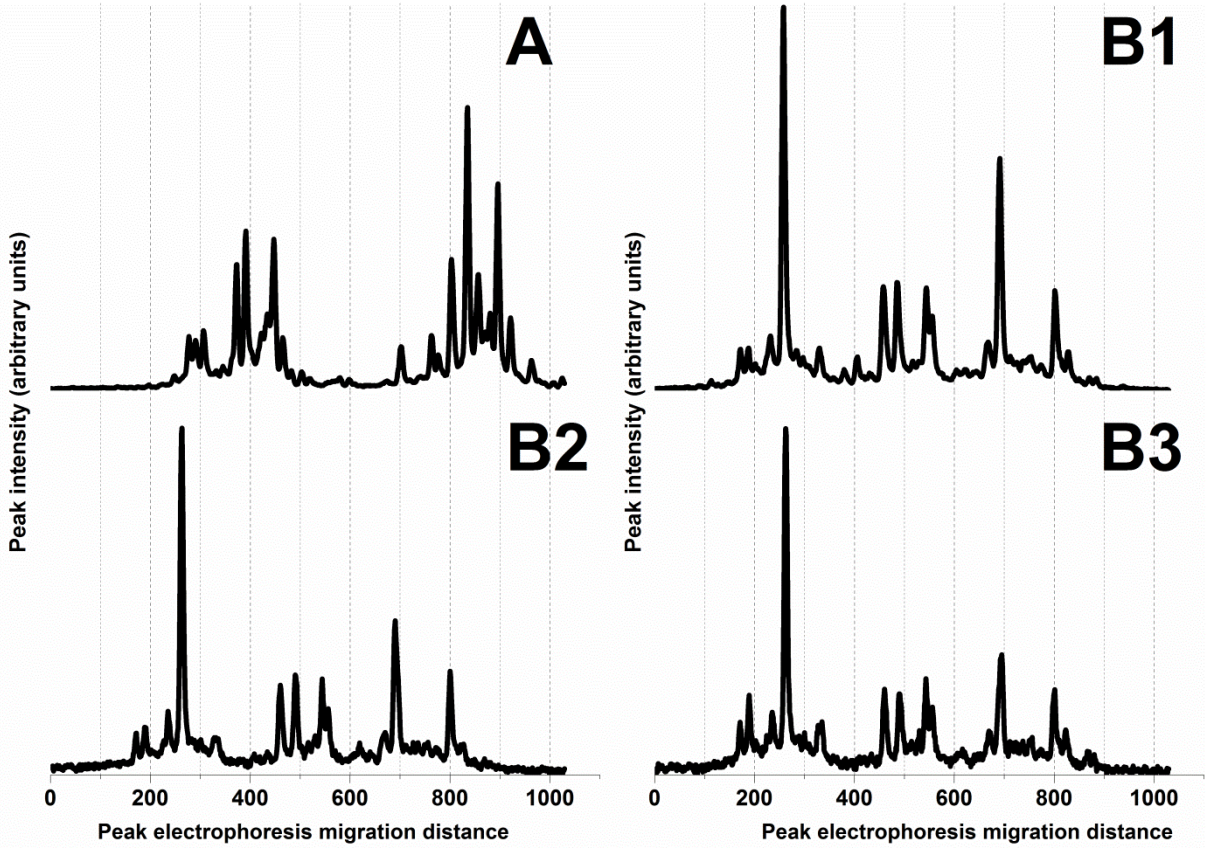

Fig. S2 - CE-SSCP profiles based on 16S rRNA gene fragments retrieved from (A) the sediments used as inoculum and (B1-3) the electroactive biofilm enriched during more than 100 days of MEC continuous operation.

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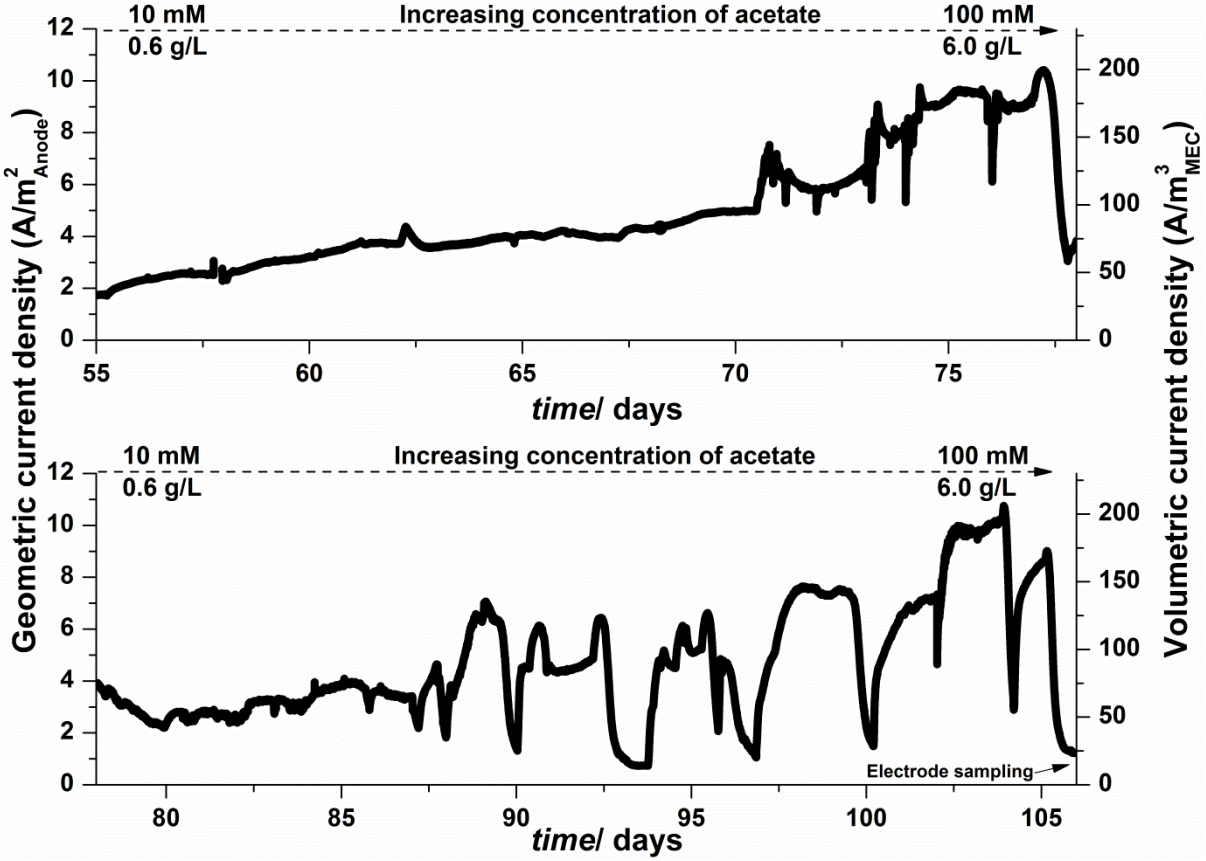

Fig. S3 - Phase 3: increasing acetate concentration test during chronoamperometric (at 200 mV vs. SCE) continuous operation of the liter scale up-flow MEC used throughout the present study. With the same electroactive biofilm within the MEC two replicates were conducted. As it can be seen, for the 2nd replicate sudden drops in current density production were observed. This was due to the salt contained in the medium that blocked the tubing used to feed the MEC as per our observations.

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Table S1 - Selected δ-proteobacterial sequences from the 16S rRNA-based phylogenetic tree (The All-species Living Tree: http://www.arb-silva.de/projects/living-tree/).

Accession number	Family	Organism name	Sequence length/ bp
AF418174	Desulfarculaceae	*Desulfarculus baarsii*	1522
AY464939	Desulfomicrobiaceae	*Desulfomicrobium thermophilum*	1518
AF418176	Desulfobacteraceae	*Desulfotignum balticum*	1505
U48243	Desulfovibrionaceae	*Desulfovibrio halophilus*	1547
DQ365924	Desulfovibrionaceae	*Desulfovibrio marinus*	1524
AAEW02000008	Desulfuromonadaceae	*Desulfuromonas acetoxidans*	1558
AY835392	Desulfuromonadaceae	*Desulfuromusa ferrireducens*	1491
DQ309326	Geobacteraceae	*Geoalkalibacter ferrihydriticus*	1504
EU182247	Geobacteraceae	*Geoalkalibacter subterraneus*	1555
U13928	Geobacteraceae	*Geobacter sulfurreducens*	1408
AY187303	Geobacteraceae	*Geopsychrobacter electrodiphilus*	1493
CP000758	Brucellaceae	*Ochrobactrum anthropi*	1476
AB498815	Bradyrhizobiaceae	*Rhodopseudomonas palustris*	1484
AY631277	Peptococcaceae	*Thermincola ferriacetica*	1443
AF005251	Shewanellaceae	*Shewanella oneidensis*	1389

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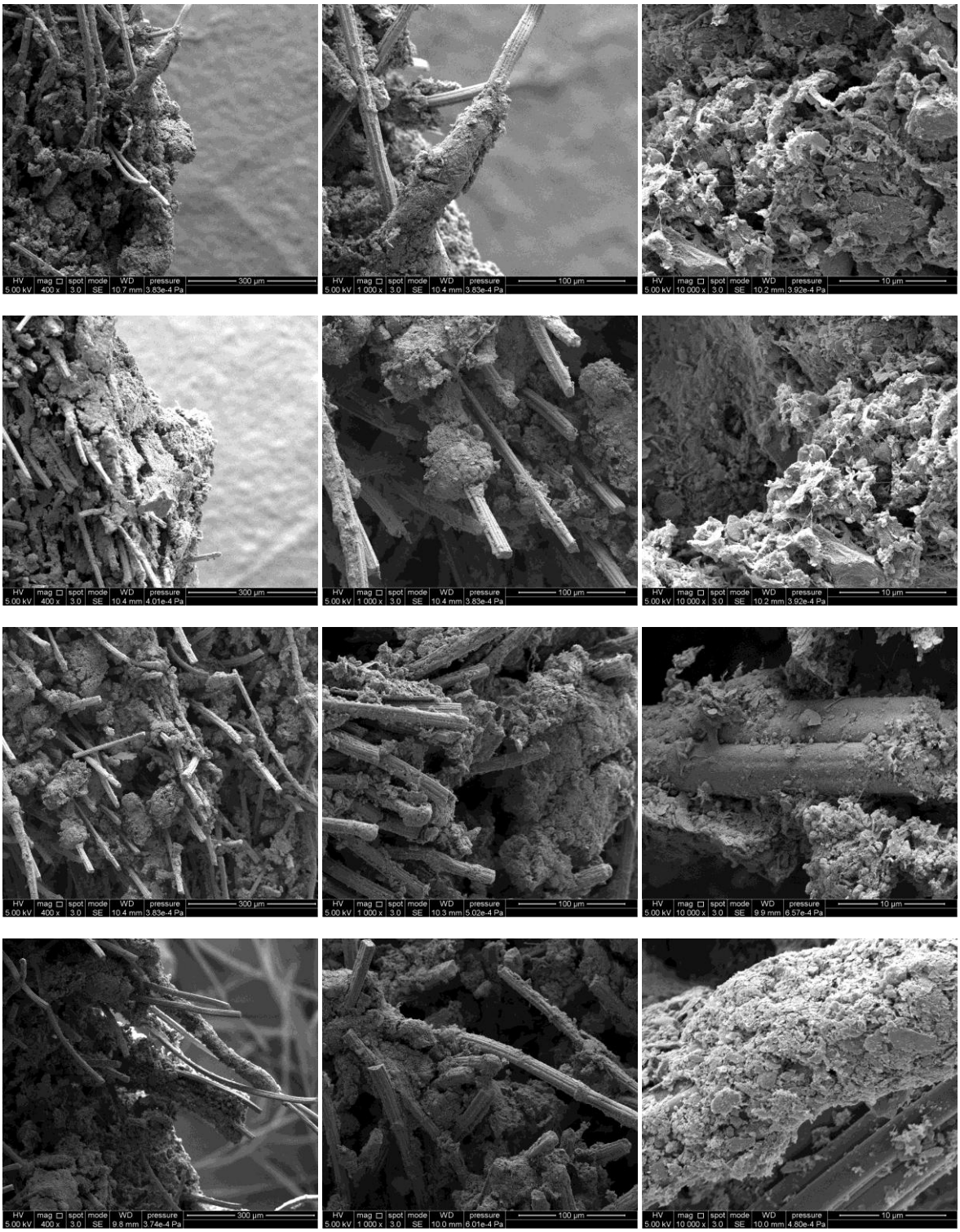

Fig. S4 - Additional exemplary scanning electron microscopy pictures of the anodic electroactive biofilm observed after more than 100 days of MEC continuous operation. Scale bars (in µm) are indicated in the bottom of the picture for left (300), middle (100) and right (10) columns.

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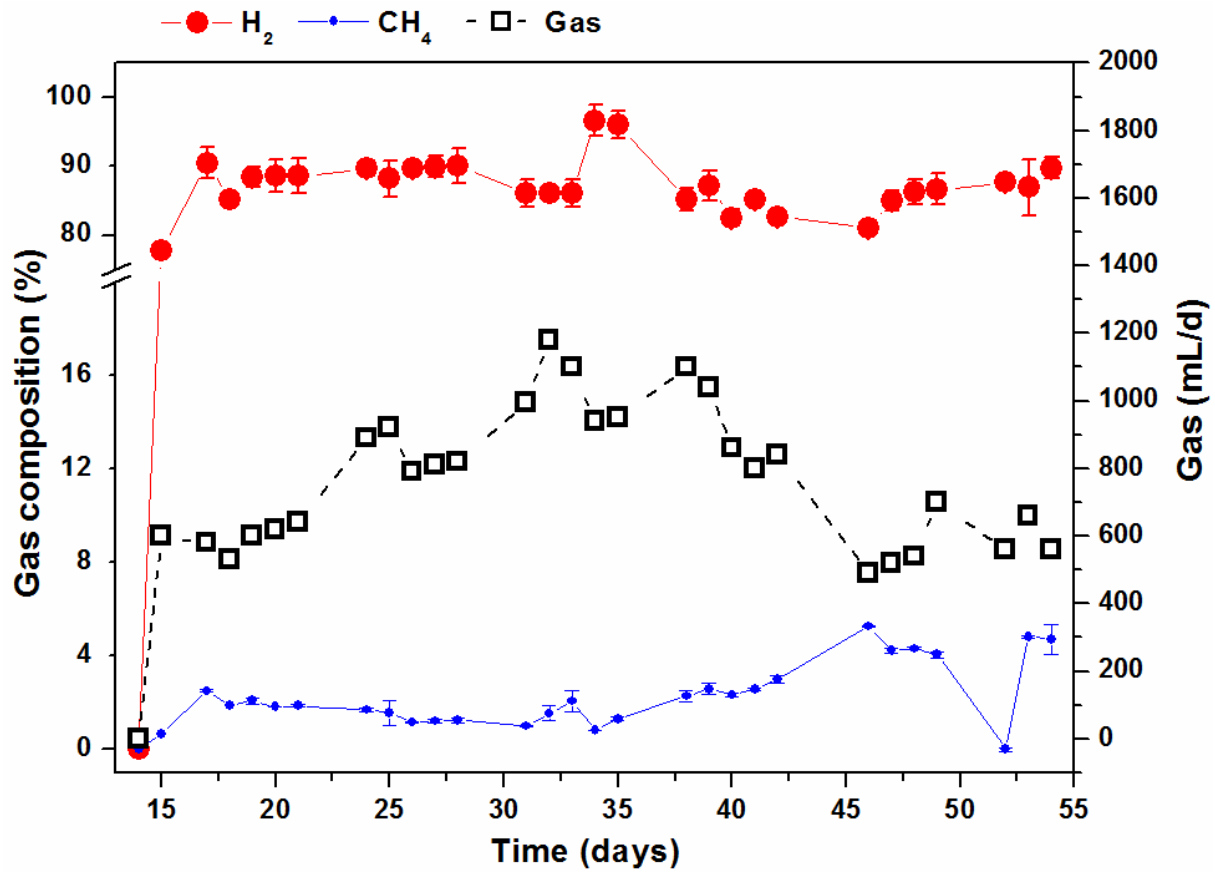

Fig. S5 - Detailed gas volume and composition sampled at the cathode chamber during Phase I.

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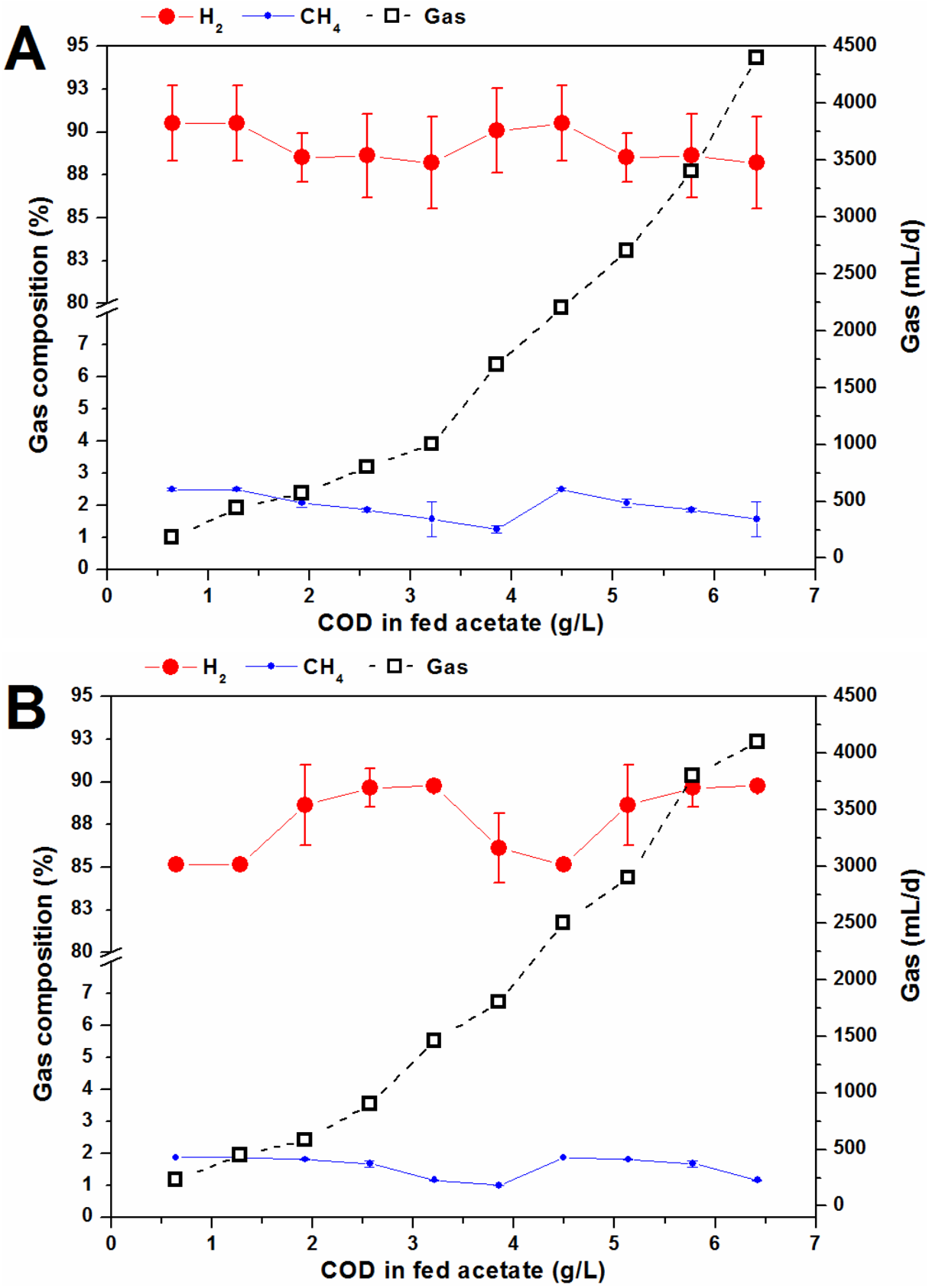

Fig. S6 - Gas volume collected and composition sampled at the cathode chamber during Phase III. The average and standard deviation of two daily measurements is shown.

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Table S2 - Substrate concentration fed to the MEC at each experimental Phase.

Acetate (mmol/L)	Acetate (g/L)	COD in fed acetate (g/L)	Phase I	Phase II	Phase III
10	0.6	0.64	x		x
15	0.9	0.96	x		
20	1.2	1.28	x	x	x
30	1.8	1.93			x
40	2.4	2.57			x
50	3.0	3.21			x
60	3.6	3.85			x
70	4.2	4.49			x
80	4.8	5.14			x
90	5.4	5.78			x
100	6.0	6.42			x

3. Benefit of producing H_2 by poising the anode potential in comparison to other strategies: i) use of a fixed cell voltage or ii) use of a poised cathode potential

With respect to the different operational conditions used in MEC studies, in the literature three types can be found from the electrochemical perspective independently if the anode and/or the cathode are mainly biologically or chemically catalyzed.

Type 1 or 2: the anode or the cathode potential is constantly controlled by a potentiostat. Such potentiostatic method provides the certainty that at least one of the electrodes is constantly fixed at a decided potential. Thus, the observed reactions occur thanks to that specific applied potential.

On the other side, in type 3 the whole MEC potential is constantly controlled by a power source without clearly being able to fix the potential at which available substrates in the medium might be oxidized or reduced at the electrodes (Batlle-Vilanova et al. 2014, Nam et al. 2011).

4. Abiotic cathode versus biotic cathode

Although the use of bio-cathodes (instead of abiotic-cathodes) in MECs shows considerable potential (Batlle-Vilanova et al. 2014), the present study was part of a project aiming at coupling two biotechnological processes: dark fermentation and microbial electrolysis. Thus, we decided to use a configuration in which we could microbiologically oxidize certain

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substrate at the anode and chemically use those electrons at the cathode to produce H2. Such configuration allowed us to well control the experimental conditions at the anode.

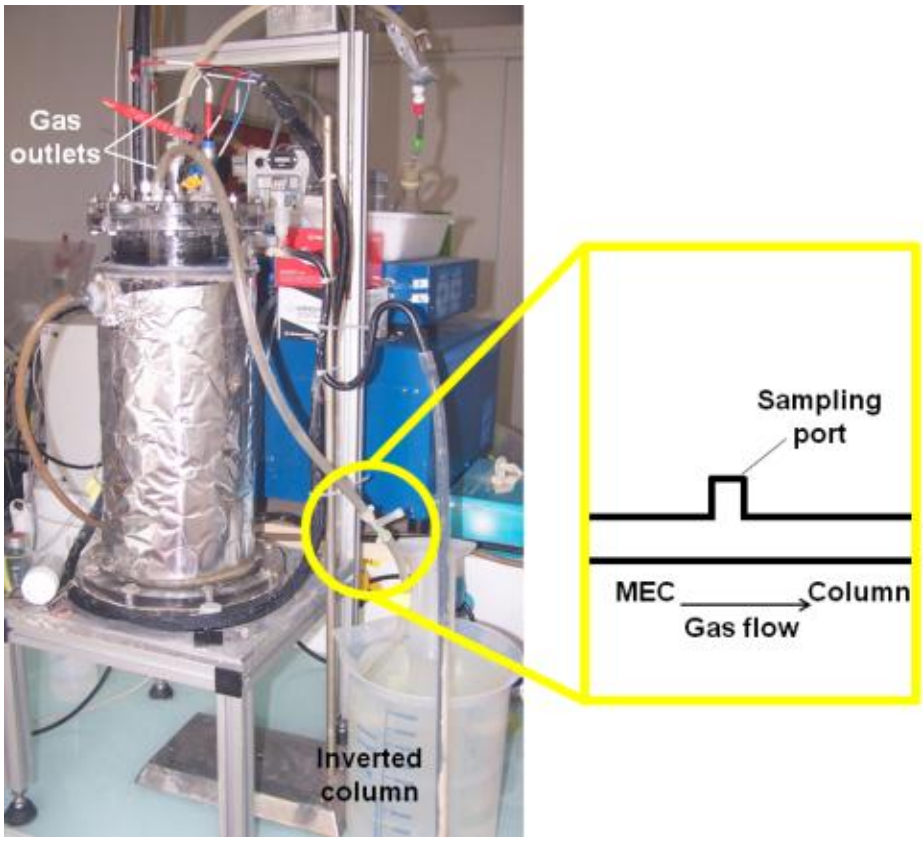

Fig. S7 - Gas produced in the system either at the cathode or anode chamber was collected in an inverted column. In between the MEC and the inverted column a sampling port was installed to analyze gas composition by chromatography.

5. Use of cyclic voltammetry

Cyclic voltammetry (CV) was mainly used to i) know the possible electron transfer mechanism (ETM) employed by the anodic biofilm and ii) demonstrate the formation of a mature biofilm (data not shown in the first version of the manuscript). We have used a previously applied approach to recognize the formation of a mature biofilm by comparing the shape of the bare electrode CV and the one after three cycles of chronoamperometry (Fig. S8). Additionally, we have plotted the 1st derivative of a CV of a mature biofilm to calculate the formal potential (Ef) which indicates the potential at which the biofilm likely transfer electrons to the electrode material (Carmona-Martínez et al. 2011, Carmona-Martínez et al. 2012, Carmona-Martínez et al. 2013).

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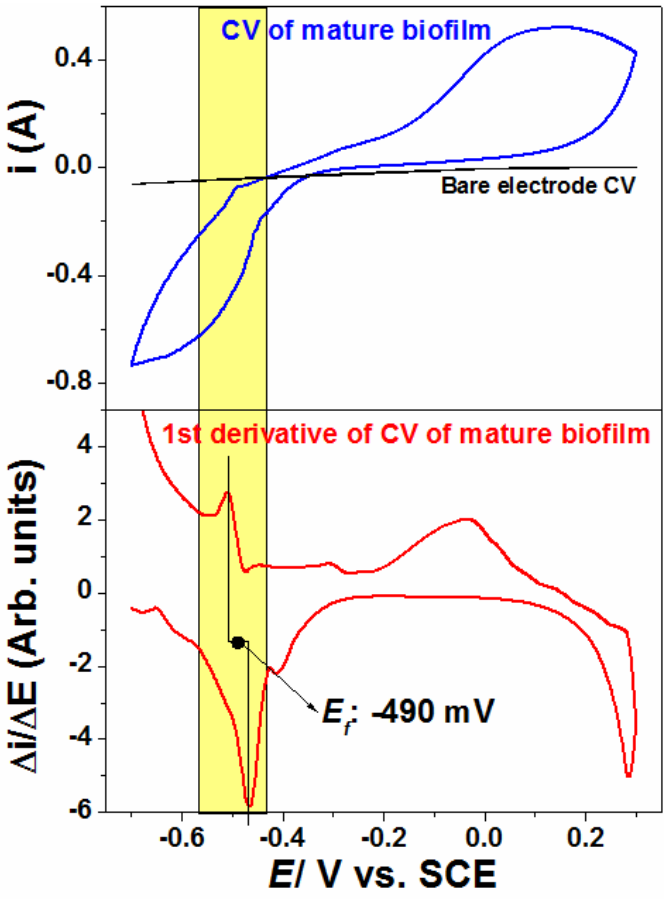

Fig. S8 - Cyclic voltammetry applied to the analysis of the anodic biofilm.

Table S3 - Recently published reports on scalable MECs treating real domestic wastewater.

$E_{app.}$ (V)	HRT (h)	MEC volume (L)	T (°C)	H_2 (%)	CE (%)	COD removal (%)	j (A/m^2)	H_2 ($m^3/m^3 \cdot d$)	Reference
1.1	24	120	16-22	100	55	33.7 ± 54.0	0.3	0.015	(Heidrich et al. 2013)
1.0	96	2	20	N.R.	24	80	0.22	0.022	(Gil-Carrera et al. 2013)
1.1	24	100	1-22	100	41	Below standards	21.3	0.006	(Heidrich et al. 2014)
0.7	42	3	19	80	238	92	0.36	0.015	(Escapa et al. 2015)
0.2	12	4	37	90	10	70	10.6	0.900	This study

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