1   **Microbial community dynamics in two-chambered microbial fuel cells: Effect of**

2   **different ion exchange membranes**

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1 **Abstract**

2 **BACKGROUND:** The utilization of different kinds of ion exchange membrane is a

3 common practice in bioelectrochemical systems such as two-chambered microbial fuel

4 cells (MFCs). However, little is known on the effect of the membrane materials on the

5 anodic microbial community diversity.

6 **RESULTS:** The effect of two cationic and one anionic exchange membranes (Nafion

7 N-117, Ultrex CMI-7000, and Ultrex AMI-7000) on the microbial community dynamics

8 of *Eubacteria* and *Archaea* has been assessed in two-chambered MFCs. The

9 experimental results indicated that the eubacterial community in the anodic chamber

10 was not affected by the membrane materials, being predominant populations of

11 *Bacteroidetes* (*Porphyromonadaceae*) and *β-proteobacteria* (*Alcaligenaceae* and

12 *Comamonadaceae*). On the other hand, the archaeal counterpart appears to be highly

13 dependent on the type of membrane used, as it was evidenced by the selective

14 enrichment of *Methanosarcina* sp. in the MFC equipped with the membrane Nafion N-

15 117 which was the MFC that showed the highest current production.

16 **CONCLUSIONS:** The results obtained in the present study suggest that membrane

17 materials affect archaeal diversity whereas both anodofilic eubacteria and methanogenic

18 archaea populations could play an important role on the overall MFC process

19 performance.

20

21 **Keywords:** Bioelectrochemical system (BES), Microbial fuel cell (MFC), Ion exchange

22 membrane (IEM), Eubacteria, Archaea, methanogens.

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1. INTRODUCTION

A microbial fuel cell (MFC) is a bioelectrochemical system (BES) designed for the direct production of electricity using microorganisms as catalysts. This biotechnology represents a promising approach for the valorisation of wastewaters and organic waste as a renewable energy source.[1,2] In a MFC, the chemical energy available in organic substrates is directly harvested in an external circuit as free flowing electrons. The efficiency of this process is, therefore, potentially higher than that of other bioconversions such as methane and hydrogen fermentations.[3]

Many different MFC configurations have been assayed,[4] but the designs based on a double chamber are rather common.[4-8] A conventional two-chambered MFC is formed by an anaerobic anodic and an aerobic cathodic chambers separated by an ion exchange membrane (IEM). The main function of the membrane is to keep the soluble components of both compartments apart, while allowing the permeation of protons from the anodic to the cathodic chamber. The ionic balance is then closed by the flow of electrons from the anode to cathode through an external circuit.

Microorganisms from the anodic chamber act as catalysts by oxidizing organic substrates and transferring the released electrons to the external circuit. This transfer can be achieved by means of different strategies: (*i*) directly *via* the physical contact with the anode (cell adhesion or through nanowires); (*ii*) mediated through exogenous redox chemical mediators, or microbial secondary metabolites (chemical shuttles); and (*iii*) mediated through microbial primary metabolites formed during anaerobic respiration or fermentation.[9] The protons produced during the biological oxidation in the anodic chamber migrate to the cathodic chamber through the IEM, where they usually combine

with oxygen and electrons to form water. Alternatively, chemicals such as ferricyanide have been used as final electron acceptor.[4,7]

As aforementioned, the IEM is a critical element in two-chambered MFCs as it maintains separation between the electron donor and acceptor while facilitating the flux of protons from the anodic to the cathodic compartments. In addition, the migration of larger ionic species and organic molecules and, especially, the diffusion of oxygen (or other electrolytes) from the cathodic to the anodic compartments, which would impair the overall process efficiency, is also prevented. Different membrane materials with specific physicochemical properties have already been assayed. Nafion, a sulfonated tetrafluoroethylene copolymer, has widely been used as proton exchange membrane (PEM) due to its high selectivity towards protons.[10] However, its relatively high price has prompted the use of less specific but structurally stronger cation exchange membranes (CEMs).[6] The use of PEMs and CEMs may still result in the migration of positive charges (cationic species) other than protons, resulting in an increased pH in the cathodic chamber and a decreased MFC performance.[11] Alternatively, anion exchange membranes (AEMs) and ultrafiltration membranes have been proposed as feasible alternatives.[6] Therefore, the utilization of both PEM and AEMs is an issue of growing up interest on bioelectrochemical research.[12,13]

The microbial ecology in the anodic chamber of a MFC may be significantly different from a methanogenic reactor albeit both are running anaerobically. On this regard, it is interesting to study the microbial community structure concerning the presence of exoelectrogenic microorganisms,[14-16] either suspended in the liquid bulk or attached in the biofilm.[17]

So far, there are two well-known bacterial genera which present exoelectrogenic activity in pure culture, i.e., *Shewanella* [18] and *Geobacter*.[19] However, the power density achieved in most of the experiments working with pure cultures turn out to be lower than those collected in mixed cultures.[20] These results reinforce the idea that increased electricity generation could be attributed to synergistic interactions within the microbial community. Namely, there could be microorganisms that do not exchange directly electrons with the electrode, but could be setting interactions between other members of the microbial community and be playing a crucial role in the operation of a MFC.

Recent studies have reported presence of archaeal cells attached to the biofilm in the anode of MFCs,[14,21,22] suggesting that they might play a role in the electron transfer process. Franks *et al.*[23] have suggested that the development of biofilms with exoelectrogenic activity may be due to syntrophic interactions between eubacteria and methanogenic archaea. Yet, conversely to the biomass of anaerobic digesters, little is known on the syntrophic interactions between bacterial and archaeal populations in BES, concerning their distribution and role in the anode of a MFC in relation to operational and design reactor parameters.

The aim of this research was to evaluate the effect of three different materials (Nafion N-117, Ultrex CMI-7000, and Ultrex AMI-7000) as IEM in the performance of two-chambered MFCs. Nafion N-117 has been widely used with good results on MFCs performance, however it is an expensive material when scaling-up of bioreactors is needed. Therefore, it is interesting to test other alternative membrane materials (cation and anion exchange membrane more economically, and compare their performances on electricity productions and its potential effects on microbial community dynamics and

1 structure. The effect of the membrane physicochemical properties on the anodic

2 microbial community structure, both in the biofilm and supernatant cells, was also

3 assessed by culture-independent molecular methods. In this work we also study the

4 interactions between eubacteria and archaea in order to gain new insights into the

5 microbial processes that potentially could govern the electron transfer in the anodic

6 compartment.

7

2. MATERIALS AND METHODS

2.1. MFC reactors

10   Two-chambered MFC reactors consisted of two Plexiglas flat plates (88×65

11 mm) bolted together were used in this research. These external plates were framed so

12 that they formed two chambers separated by an IEM fitted using neoprene gaskets

13 (NCBE, University of Reading, UK). Both the anode and the cathode electrodes were

14 made of carbon fibre tissue and were connected to an external resistance of 1000 Ω

15 through an electric circuit. The IEM had an effective surface area of 12 cm^2 exposed to

16 either compartment. Three different IEM materials were investigated: Nafion N-117

17 (DuPont Co., Wilmington, DE, USA) as PEM, Ultrex CMI-7000 (Membranes

18 International Inc., Ringwood, NJ, USA) as CEM; and Ultrex AMI-7000 (Membranes

19 International Inc.) as AEM. Experiments were performed at room temperature and by

20 duplicate. Thus, a total of six MFC reactors were set-up and operated in parallel.

21   The anodic chamber was filled with 8 mL of a mineral medium, 1 mL of

22 inoculum, and 1 mL of a sodium acetate solution (2.95 g/L9) being 0.1M the final

23 concentration in the anodic chamber. The mineral medium was prepared according to

24 Kennes et al.,[24] containing (per litre): 4.5 g KH$_2$PO$_4$, 0.5 g K$_2$HPO$_4$, 2 g NH$_4$Cl, 0.1 g

1 $MgSO_4 \cdot 7H_2O$, 1mL of a trace mineral solution, and 1mL of a vitamin solution. The

2 experiment lasted 90 days, during which the anodic chamber was fed with the acetate

3 solution (1 mL of anolyte was replaced by 1 mL pulse of acetate solution 2.95 g/L in

4 mineral medium) each time the voltage decreased to the baseline value. The cathodic

5 chamber was filled up with 10 mL of a chemical solution containing (per litre): 16.5 g

6 $K_3Fe(CN)_6$ as final electron acceptor and 4.5 g KH_2PO_4 + 0.5 g K_2HPO_4 as phosphate

7 buffer.

8 **2.2. Inoculum**

9 The MFC anodic chamber was inoculated with 1 mL of digestate (49 g COD/L

10 and 2.5 g NH_4^+-N/L) from a bench-scale mesophilic methanogenic continuously stirred

11 reactor fed with slaughterhouse waste under hydraulic retention times of 20-30 days,

12 organic loading rates of 2-3 g COD/L/d, and nitrogen loading rates of 0.08-0.14 g

13 N/L/d, as previously described by Rodríguez-Abalde *et al.*[25]

14

15 **2.3. Electrochemical characterization**

16 The voltage in the external circuit of the MFC was recorded every 10 minutes

17 using a data acquisition unit (Mod. 34970A, Agilent Technologies, Loveland, CO,

18 USA). The current density (*I*) was then calculated according to the Ohm's law (eq. 1),

19 and the power density (*P*) was calculated with (eq. 2),

20 $$I=V/R \qquad\qquad (eq.\ 1)$$

21 $$P=I^2 \cdot R \qquad\qquad (eq.\ 2)$$

22 where *I* stands for current density (mA), *V* stands for the voltage (mV), *R* is the external

23 resistance *(Ω), P* is the power density (mW/m^2) and *A* stand as the electrode surface

24 area (m^2) and *P* stands for power density (mW/m^2).

Polarization curves (*P* versus *I*) were performed at different moments of the

experiment to estimate the enrichment of the exoelectrogenic community and calculate

the maximum value of *P,* which is obtained with the internal resistence *(Ω)* of the

system. The procedure to obtain a polarization curve was as follows: after leaving the

system 1 hour in open circuit, the circuit was closed and the external resistance was

varied in the range from 30000 to 1.2 Ω. Upon the connexion of each resistance, the

system was left for stabilization during 30 min before recording the voltage data.

The Coulombic efficiency (*CE*), defined as the fraction of electrons recovered as

current versus the maximum theoretical recovery from the substrate oxidation,[4] was

calculated using data collected after acetate pulses and using (eq. 3),

$$CE=C_p/C_{Ti}*100 \qquad\qquad (eq.\ 3)$$

where *CE* is the Coulombic efficiency (%)*,* C_p is the total number of Coulombs

estimated by integrating the electric current over time and C_{Ti} is the theoretical amount

of Coulombs that can be produced from acetate, calculated assuming total removal of

the acetate added, similarly as previously described by Liu and Logan.[26]

2.4. Scanning Electron Microscopy (SEM)

At the end of the experiments,the occurrence of different kind of material

accumulations on the IEM surface was also assessed. The used IEMs were observed

with a Scanning Electron Microscope (SEM) (mod. Quanta 200, FEI Co., Hillsboro,

OR, USA) operated at 15 kV and high vacuum. The samples were placed on stubs using

double-stick tape and coated with carbon. The surface of the samples on the slides was

observed through secondary electrons (SE) and back-scattered electrons (BSE) imaging,

and X-ray energy dispersive spectroscopy (EDS). Surface IEMs measurements were

1 performed at Nanometric unit in Scientific and Technological Centers of the University

2 of Barcelona (CCiTUB).

3

4 **2.5. Denaturing gradient gel electrophoresis (DGGE) molecular profiling**

5   Culture-independent molecular techniques were applied in order to analyse the

6 eubacterial and archaeal microbial communities in the digestate used as inoculum,

7 supernatant in the anodic chamber, and anodic biofilm formed in the carbon fibre tissue

8 working as electrode. Samples from the MFC anodic chamber supernatant were taken at

9 7, 20, and 60 days of operation, when an electricity production peak was observed

10 (peaks number 1, 2, and 3, respectively), while anode biofilms were sampled at the end

11 of the experimental phase from carbon fibre tissue material in the anode compartment

12 after 90 days.

13   Total DNA was extracted in triplicate from known volumes/weights of each

14 sample with the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad,

15 CA, USA), according to manufacturer's instructions. Universal eubacterial forward

16 F341 and reverse R907 primers were used to amplify the hypervariable V3-V5 region

17 from the *16S rRNA* gene by the polymerase chain reaction (PCR), as previously

18 reported.[27] For archaeal population a NESTED-PCR approach was performed by using

19 the primer pairs Arch0025/R1517 and F344-R915-GC for the PCR and the nested

20 reaction, respectively.[28] All PCR reactions were carried out in a Mastercycler

21 (Eppendorf, Hamburg, Germany) and each reaction mix (25 μL mix/reaction) contained

22 1.25 U of Ex TaqDNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan), 12.5 mM

23 dNTPs, 0.25 μM of each primer, and 100 ng of DNA.

1   The PCR amplicons (20 µL) were loaded in an 8% (w/v) polyacrylamide gel

2   (0.75 mm thick) with a chemical denaturing gradient ranging from 30% to 70% (100%

3   denaturant stock solution contained 7 M urea and 40% (w/v) of formamide). The

4   electrophoresis was carried out in a DGGE-4001 system (CBS Scientific Company Inc.,

5   Del Mar, CA, USA) at 100 V and 60°C for 16 h in a 1x TAE buffer solution (40

6   mMTris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4).[29] The DGGE gels were stained

7   in darkness for 45 min with 15 mL of 1x TAE buffer solution containing 3 µL of

8   SYBR® Gold 10,000x (Molecular Probes, Eugene, OR, USA). The gels were scanned

9   under blue light by means of a blue converter plate (UV Products Ltd., Cambridge, UK)

10   and a transilluminator (GeneFlash, Synoptics Ltd., Cambridge, UK). Predominant

11   DGGE bands were excised with a sterile filter tip, suspended in 50 µL of molecular

12   biology grade water, and stored at 4ºC overnight. The resuspended bands were

13   subsequently reamplified by PCR as described above. Sequencing was accomplished by

14   using the ABI Prism Big Dye Terminator Cycle-Sequencing Reaction Kit v.3.1 and an

15   ABI 3700 DNA sequence (both Perkin–Elmer Applied Biosystems, Waltham, MA,

16   USA), according to manufacturer´s instruction.

17   Sequences were processed by BioEdit software package v.7.0.9 (Ibis

18   Biosciences, Carlsbad, CA, USA) and aligned by BLAST basic local alignment search

19   tool (NCBI, Bethesda, MD, USA) and the Naïve Bayesian Classifier tool of RDP

20   (Ribosomal Database Project) v.10 (East Lansing, MI, USA) for the taxonomic

21   assignment. After the alignment, Bellerophon v.3 (GreenGenes, Berkeley, CA, USA)

22   was used to eliminate chimeric sequences. The eubacterial and archaeal *16S rRNA* gene

23   nucleotide sequences determined in this study were deposited in the Genbank (NCBI)

24   under accession numbers JQ307401-JQ307412 and JQ394939-41, JQ394943-

1   JQ394944, JQ394946-JQ394948, JQ394950-JQ394952, JQ394954, JQ394959-

2   JQ394961, JQ394964- JQ394966 for eubacterial and JQ394967-JQ394968,JQ394970-

3   JQ394971,JQ394977-JQ394979.

4   Changes on the microbial community structure were analysed by covariance-

5   based Principal Component Analysis (PCA) based on the position and relative intensity

6   of the bands present on the DGGE profiles previously digitalized. The MS Excel

7   application StatistiXL v.1.4 (Broadway, Nedlands, Australia) was used for this purpose.

8

9   **2.6. Quantitative PCR assay (qPCR)**

10   Gene copy numbers of eubacterial *16S rRNA* gene and *mcrA* gene

11 (methanogenic archaeal methyl coenzyme-M reductase) were quantified by means

12 quantitative real-time PCR (qPCR). Each sample was analyzed in triplicate by means of

13 three independent DNA extracts as elsewhere described.[30] The analysis was carried out

14 by using Brilliant II SYBR Green qPCR Master Mix (Stratagene, La Jolla, CA, USA) in

15 a Real-Time PCR System Mx3000P (Stratagene) operated with the following protocol:

16 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 30

17 s at 50ºC and 54ºC (for *16S rRNA* and *mcrA* gene, respectively), extension at 72°C for

18 45 s, and fluorescence capture at 80°C. The specificity of PCR amplification was

19 determined by observations on a melting curve and gel electrophoresis profile. Melting

20 curve analysis to detect the presence of primer dimers was performed after the final

21 extension by increasing the temperature from 55 to 95°C at heating rates of 0.5°C each

22 10 s. Image capture was performed at 82°C to exclude fluorescence from the

23 amplification of primer dimers. Each reaction was performed in a 25 µL volume

24 containing 2 µL of DNA template, 200 nM of each *16S rRNA* primer, 600nM of each

1   *mcrA* primer, 12.5 µL of the ready reaction mix, and 30 nM of ROX reference dye. The

2   primer set for eubacterial population was 519FqPCR (5'-

3   GCCAGCAGCCGCGGTAAT-3') and 907RqPCR (5'-CCGTCAATTCCTTTGAGTT-

4   3'). The primer set for archaeal *mcrA* gene was ME1F (5'-

5   GCMATGCARATHGGWATGTC-3') and ME3R (5'-

6   TGTGTGAASCCKACDCCACC-3');[31] both primer pairs were purified by HPLC. The

7   standard curves were performed with the following reference genes: *16S rRNA* gene

8   from *Desulfovibrio vulgari*s ssp. *vulgaris* ATCC 29579, and *mcrA* gene fragment

9   obtained from *Methanosarcina barkeri* DSM 800, both inserted in a TOPO TA vector

10   (Invitrogen Ltd., Paisley, UK). All reference genes were quantified by NanoDrop 1000

11   (Thermo Scientific). Ten-fold serial dilutions of known copy numbers of the plasmid

12   DNA in the range 10 to 10^8 copies were subjected to a qPCR assay in duplicate to

13   generate the standard curves. The qPCR efficiencies of amplification were greater than

14   98%. All results were processed by MxPro QPCR Software (Stratagene).

15

16   **3. RESULTS AND DISCUSSION**

17   **3.1. Electrochemical activity**

18   The electricity production capacity of the MFCs was compared in terms of

19   voltage generation and polarisation curves. A sharp increase in the voltage was

20   observed in all the reactors after an acetate feeding pulse, but the voltage decrease

21   patterns were different depending on the type of membrane (Fig. 1). The MFCs

22   equipped with Nafion N-117 exhibited a greater electricity production than those built

23   with Ultrex CMI-7000 and Ultrex AMI-7000 membranes, with a *CE* for one acetate

1 feeding cycle of 13%. The *CE* in the MFCs using CMI-7000 and AMI-7000 membranes

2 was 5.7% and 6.7%, respectively.

3 Similarly, the highest voltage and power density when performing the

4 polarization curves were achieved again with the N-117 membrane, with 433 mV and

5 71 mW/m^2, respectively (Fig. 2(b)). These results are in the same range than the values

6 reported elsewhere for small-sized MFCs, accounting for a similar potential but higher

7 internal resistance (2200 Ω) and lower *CE* values.[32,33] However, it is well known that

8 Nafion N-117 contains sulfonic acid groups that bind with the ammonia present in the

9 anolyte. Hence, this membrane could display a low stability and trap free nitrogen.[10]

10 Results of the MFC equipped with the N-117 membrane were followed by those of the

11 MFC equipped with the CMI-7000 membrane. Finally, a significantly lower electricity

12 production was observed with the AMI-7000 membrane compared to the other two

13 membranes tested in this study (Fig. 1 and 2(a)). Although little is known on the

14 performance of anion exchange membranes such as AMI-7000, there are some studies

15 reported in literature that show even better results with AEMs than with CEMs.[8,15]

16 Depletion of the current density production was observed in all the MFCs after

17 10 acetate feeding cycles (two months). Membrane fouling and clogging could be

18 responsible for this progressive decay in the current density production. In order to have

19 more insight of this phenomenon, the IEMs were analyzed by means of SEM and EDS.

20 The results showed the predominance of Fe and K crystallites in both membrane sides

21 and for the three materials tested (Figure S1 supplementary data). It is well known that

22 CEMs such as N-117 or CMI-7000 may be permeable to certain chemicals such as

23 oxygen, ferricyanide and other ions.[4] In the present study, the N-117 membrane

1   apparently had less precipitates attached and maintained the activity of the MFC for

2   longer time, which contrasts with results reported in some previous works.[34]

3

4   **3.2. Microbial community analysis**

5   Microbial community characterization (*Eubacteria* and *Archaea* domains) was

6   performed by means of DGGE profiling (*16S rRNA* genes) and qPCR technique (*16S*

7   *rRNA* and *mcrA* genes) on samples encompassing three acetate feeding cycles. The

8   obtained DGGE results (Fig. 3(a) and Fig. 4) showed a significant microbial population

9   shift of both eubacteria and archaea over time, concomitantly with an increment in

10   electricity production. Such microbial community changes, as observed in the DGGE

11   patterns, might be related to the adaptation of the initial inoculum, obtained from an

12   anaerobic digester, to the presence of an external electric circuit. Population dynamics

13   in eubacteria were rather independent on the type of membrane used in MFC

14   experiments. Principal Component Analysis (PCA) on parameterized eubacterial *16S*

15   *rRNA* DGGE profiles (Fig. 3(b)), showed that the most significant changes in the

16   microbial community structure coincided with the second and third peak of voltage

17   production. Yet, it is noteworthy that the most differentiated microbial community has

18   been observed in the MFC equipped with the N-117 membrane (third peak) (Fig. 3(b)),

19   which it also displayed the best performance. Band 36 (Fig. 3(a)) belong to *Mollicutes*

20   (*Acholeplasmataceae*) has been identified only for the sample corresponding to the third

21   peak of N-177 sample.

22   Conversely, changes on the archaeal microbial community over time were strongly

23   dependent on the membrane material (Fig. 4 and Fig. 5). The membrane materials are

24   different, such as N-117 membrane contains sulfonic acid groups which could directly

1   affect the archaea metabolisms. It is noteworthy that ribotypes closely related to the

2   methanogenic genus *Methanosarcina* (DGGE band 1 (Fig. 4) and DGGE bands 27, 35,

3   36, and 39 (Fig. 5) were only found in MFC equipped with cationic membranes (N-117

4   and CMI-7000), whereas ribotypes belonging to Methanosateaceae where detected both

5   in anionic and cationic membranes (bands 10, 13, 15, 38). Such important occurrence of

6   Methanosarcina detected in cationic membranes-MFC was also coincident with a higher

7   electricity production in Nafion N-117-MFC. In addition, the *mcrA* gene copy numbers

8   quantified by qPCR revealed a high abundance of methanogenic archaea in the three

9   MFC systems, representing the 6-20% in relation to the total eubacterial *16S rRNA* gene

10   copy numbers (Fig. 7). In addition, it is noteworthy, that Nafion-MFC harboured the

11   highest methanogenic population on the anode biofilm (10^9 *mcrA* gene/g_{dw} anode)

12   accounting for 17% of total population, compared with CMI-MFC (7%) and AMI-MFC

13   (11%). Previous studies reported also a high prevalence of methanogenic archaea close

14   to the anode on MFC and MEC bioelectrochemical systems.[35,36] Although

15   methanogenic archaea could compete for the electrons and have been pointed as

16   responsible for the low *CE*, they could play a role in the establishment and function of

17   an anodofilic biofilm by improving the bioavailability of cofactors, and other molecules

18   such as electron shuttles enhancing electron transfer among microorganisms.[37,38]

19   It is noteworthy that no significant differences were found in relation to the

20   microbial community structure of supernatant (planktonic cells) and biofilm-forming

21   eubacteria (Fig. 6), in agreement with a recent work from Bonmatí *et al.*[39] where the

22   predominant bands in both supernatant and biofilm belonged to the same phylum.

23   Contrary, the archaeal communities from the biofilm and the supernatant were

24   significantly different and dependent on the membrane material (Fig. 5). Such spatial

differentiation in the archaeal community composition could be explained by the enrichment of archaea in contact with the anode, which could encompass specialized representatives both in cooperation and in competence with exoelectrogenic eubacteria. However, further research is needed in this field to confirm such potential interaction between eubacteria and targeted methanogens belonging to Methanosarcinaeae and Methanosaetaceae in our MFC reactors.

Sequences of most predominant eubacterial ribotypes from DGGE bands (Fig. 3(a) and 6) are presented on Tables 1 and 2. The most predominant eubacterial ribotypes found in the supernatant and the biofilm are associated to *Bacteroidetes* (*Prophyromonadaceae*) and *β-Proteobacteria* (*Alcaligenaceae* and *Comamonadaceae*). Representatives of these microbial groups have previously been described as being able to exchange electrons with an electrode.[40] Within the *β-Proteobacteria* class, we observed several bands on the DGGE profiles with sequences belonging to the *Alcaligenaceae* and *Comamonadaceae* families. Representatives of these families have been previously described as electrochemically active in studies also performed with methanogenic sludge as inoculum.[22,41]

Regarding the archaeal DGGE profiles (Fig. 4 and Fig. 5) the phylogenetic assignment on basis of the DNA sequence homology searches has been summarized in Table 3. The observed diversity of archaea was significantly lower than that of eubacteria. It is remarkable that all methanogenic sequences belonged to the *Methanosaetaceae*, *Methanomicrobiaceae* and *Methanosarcinaceae* families. There is one particular ribotype sequence belonging to the *Methanosarcinaceae* which is apparently enriched over time in the reactors equipped with cationic membrane (band 27) (Fig. 5), and specially with N-117 membrane (band 1) (Fig. 4). Yet, those bands

belonging to *Methanosaetaceae* and *Methanosarcinaceae* were found to be predominant in the biofilm for the three studied MFC configurations. It is noteworthy that methanosarcinaceae are specially enriched in MFC equipped with cationic membranes. So far, relatively few studies have focussed on the archaea that are present in anode compartment of a MFC reactor. Also, we quantified the population by means of qPCR and we can conclude that there is a high number of methanogenic archaea, both in the biofilm (6.3 10^8 -1.5 10^9 *mcrA* gene copies g_{dw}^{-1} anode) and in the planktonic community (2.8 10^7 – 4.5 10^8 *mcrA* gene copies mL^{-1} anolyte) (Fig. 7).

The unravelled low diversity high abundance of methanogenic archaea would suggest that specific methanogenic archaea could play an important role in the MFC performance. In this regard, Chung and Okabe[14] reported FISH and SEM images where methanogenic archaea were colonizing the anode surface in concomitance with several eubacteria. Moreover, Croese *et al.*[36] reported the presence of methanogenic archaea in the bulk between electrode fibres, but in this case, they are not physically attached to the anode. Besides, it has been reported that some methanogens can directly reduce solid iron Fe (III) oxide,[5] pointing out the possibility that methanogenic archaea could contribute to electricity generation by means of exoelectrogenic strategies, which is being reinforced in recent experiments by Rotaru *et al.*[42] and Malvankar *et al.*,[43] where it has been reported that methanogenic archaeae, like *Methanosaeta* and *Methanosarcina* species, are capable of exchange electrons via direct interspecies electron transfer and this would have outstanding implications in the field of anaerobic digesters and MFCs.

4. CONCLUSIONS

Microbial community population profiles show a clear enrichment in specific microbial ribotypes regardless of the type of membrane tested. Concerning the electrochemical activity, different patterns depending on the type of membrane were observed. The highest power density values were obtained with the MFC equipped with the N-117 membrane. These differences indicate that the eubacterial community was not affected by membrane materials, while the archaeal counterpart appears to be highly dependent on the type of membrane used, as evidencing by the selective enrichment of *Methanosarcina* spp. in the MFC equipped with cationic membranes, especially with Nafion (N-117). The specific microbial diversity contained in the anode biofilm and the minor extent of crystallite deposition in the N-117 membrane could explain the highest potential and power density achieved with this set-up.

Based on our results it has been proposed that further studies are needed in order to better understanding synergic eubacteria and methanogenic archaea interactions in BES reactors such as MFC community.

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2 Table 1. Characteristics of the sequenced bands from Eubacterial *16S rRNA* gene based-

3 DGGE from samples obtained in the MFCs. DA: initial inoculum. 1, 2, and 3:

4 electricity production peak number (Figure 3).

5

Band	Length (bp)	Accession number	Phylogenetic group (RDP)	Reference species, strain or uncultivated microorganism (environmental source)	Accesion number	H (%)
4	480	JQ307401	*Bacteroidetes*	Uncultured (ASBR reactor treating swine waste)	GQ135359	99
				Sphingobacterium thermophilum strain CKTN2[T]	NR_108120	87
5	357	JQ307402	*Bacteroidetes*	Uncultured (mesophilic anaerobic digester)	KF147566	98
				Cellulophaga tyrosinoxydans strain VSW306	KC534369	86
10=32	425	JQ307403	*γ – proteobacteria*	*Acinetobacter* sp. WX-19	JF730216	100
			Moraxellaceae	*Acinetobacter seohaensis*[T]	AY633608	99
13	415	JQ307404	*B-proteobacteria* *Comamonadaceae*	*Comamonas testosteroni* strain BK1R	KC864773	99
16	270	JQ307406	*B-proteobacteria* *Alcaligenaceae*	*Advenella kashmirensis* strain 20rA (bioanode in MEC)	KF528154	98
				Advenella kashmirensis WT001[T]	NR_074872	97
17	440	JQ307407	*Bacteroidetes*	Uncultured (mesophilic anaerobic digester)	EU104338	98
				Paludibacter propionicigenes WB4[T]	NR_074577	90
18	499	JQ307408	*Bacteroidetes*	Uncultured (ASBR reactor treating swine waste)	GQ134808	99
				Uncultured (microbial fuel cell)	JX174653	97
19=37	471	JQ307409	*Bacteroidetes* *Porphyromonadaceae*	Uncultured (MFC with phosphate buffer and acetate)	GQ152958	99
				Proteiniphilum acetatigenes strain TB107[T]	NR_043154	97
21	432	JQ307410	*Bacteroidetes* *Bacteroidaceae*	*Bacteroides coprosuis* strain JCM 13475[T]	AB510699	99
24	361	JQ307405	*B-proteobacteria* *Comamonadaceae*	Uncultured (MFC fed with sucrose)	HM043267	100
				Comamonas jiangduensis strain YW1[T]	NR_109655	99
				C. kerstersii strain CIP 107987[T]	EU024144	99
34	438	JQ307411	*B-proteobacteria* *Alcaligenaceae*	*Alcaligenes faecalis* strain MUN1	KF843701	100
36	475	JQ307412	*Mollicutes* *Acholeplasmataceae*	*Acholeplasma parvum* strain H23M	NR_042961	92
				A. palmae strain J233	NR_029152	92

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7 The most homologous sequence and the closest phylogenetically relevant match are shown (preferably type strains[T]).

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1 Table 2. Characteristics of the bands from the *16S rRNA* DGGE eubacteria gel from the

2 following samples: D.A: initial inocula; Nafion-B: biofilm of the MFC equipped with

3 Nafion N-117; Nafion-S: supernatant of the MFC (Nafion N-117); CMI-B: biofilm of

4 the MFC (Ultrex CMI-7000); CMI-S: supernatant of the MFC (Ultrex CMI-7000).

5 AMI-B: biofilm of the MFC (Ultrex AMI-7000); AMI-S: supernatant of the MFC

6 (Ultrex AMI-7000) (Figure 5).

7

Band	Length (bp)	Accession number	Phylogenetic group (RDP)	Reference species, strain or uncultivated microorganism (environmental source)	Accesion number	H (%)
1=4	495	JQ394939	*Bacteroidetes*	Uncultured (ASBR reactor treating swine waste)	GQ134808	100
2	498	JQ394940	*Bacteroidetes* Porphyromonadaceae	Unculrured (MFC with phosphate buffer and Acetate)	GQ152958	99
				Proteiniphilum acetatigenes strain TB107^T	NR_043154	98
3	517	JQ394941	*Bacteroidetes*	Uncultured (biogas reactor)	HG007883	99
				Sphingobacterium thermophilum strain CKTN2^T	NR_108120	87
5	325	JQ394943	*Bacteroidetes* Porphyromonadaceae	Uncultured (anodic biofilm of double-chamber MFC)	JX944537	98
				Proteiniphilum acetatigenes strain TB107^T	NR_043154	95
7	476	JQ394944	*α- proteobacteria*	Brevundimonas olei strain MJ15^T	GQ250440	91
9	484	JQ394946	*Bacteroidetes*	Uncultured (ASBR treating swine waste)	GQ134808	100
16=44	516	JQ394947	*Bacteroidetes* Porphyromonadaceae	Uncultured (biofilm from electrode material in a MFC)	JQ724340	98
				Proteiniphilum acetatigenes strain TB107^T	NR_043154	96
17	522	JQ394948	*Bacteroidetes*	Uncultured (biogas reactor)	AB826041	99
21=28	516	JQ394950	*B-proteobacteria* Alcaligenaceae	*Kerstersia gyiorum* strain LMG 5906^T	NR_025669	99
22=43	484	JQ394951	*Bacteroidetes*	Uncultured (ASBR treating swine waste)	GQ134808	97
24	331	JQ394952	*Bacteroidetes*	Uncultured (biogas reactor)	HG007883	91
				Cytophaga fermentas strain NBRC15936^T	AB517712	86
26	522	JQ394954	*γ – proteobacteria*	*Pseudomonas xiamenensis* strain JD6	JQ246783	91
36	504	JQ394959	*Acholeplasmataceae Tenericutes*	*Tenericutes bacterium* P19x1ox-fac	JQ411296	93
41	344	JQ394960	*Deferribacteres*	Uncultured (anaerobic microbial consortium growing in MFC anode fed with microalgal biomass)	JN676221	86
				Geovibrio thiophilus strain AAFu3	NR_028005	87
42	453	JQ394961	*B-proteobacteria* Alcaligenaceae	*Alcaligenes faecalis* strain G	KJ000880	99
45	501	JQ394964	*B-proteobacteria* Alcaligenaceae	*Alcaligenes faecalis* strain CPO 4.0058	KF921605	99
46	357	JQ394965	*α- proteobacteria*	*Brevundimonas* sp. P10	JX908719	93
52	428	JQ394966	*α- proteobacteria* Phyllobacteriaceae	*Defluvibacter lusatiensis* strain ST39	FJ982919	96

8

9 The most homologous sequence and the closest phylogenetically relevant match are shown (preferably type strains^T).

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1 Table 3. Characteristics of the bands from the *16S rRNA* DGGE archaea gel from the

2 following samples: D.A: initial inoculums; Nafion-B: biofilm of the MFC equipped

3 with Nafion N-117; Nafion-S: supernatant of the MFC (Nafion N-117); CMI-B: biofilm

4 of the MFC (Ultrex CMI-7000); CMI-S: supernatant of the MFC (Ultrex CMI-7000).

5 AMI-B: biofilm ofthe MFC (Ultrex AMI-7000); AMI-S: supernatant of the MFC

6 (Ultrex AMI-7000) (Figure 4).

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Band	Length (bp)	Accession number	Phylogenetic group (RDP)	Reference species, strain or uncultivated microorganism (environmental source)	Accesion number	H (%)
10	488	JQ394967	*Methanomicrobia Methanosaetaceae*	*Methanosaeta concilii* strain Opfikon[T]	NR_028242	99
13=15=38	488	JQ394968	*Methanomicrobia Methanosaetaceae*	Anaerobic *methanogenic archaeon* E15-4	AJ244290	99
21=28	488	JQ394970	*Methanomicrobia Methanomicrobiaceae*	*Methanoculleus bourgensis* strain Mcu(1)	JN413087	99
22=23	491	JQ394971	*Thermolasmata*	*Thermoplasmata archaeon* Kjm51s	AB749767	100
35=36	486	JQ394977	*Methanomicrobia Methanosarcinaceae*	*Methanosarcina barkeri* strain DSM 800[T]	NR_025303	99
39=27	486	JQ394979	*Methanomicrobia Methanosarcinaceae*	*Methanosarcina soligelidi* strain SMA-21[T] *M. barkeri* strain MS[T]	NR_109423 JQ346756	98 98

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9 The most homologous sequence and the closest phylogenetically relevant match are shown (preferably type strains[T]).

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FIGURE CAPTIONS

Figure 1. Voltage produced in MFCs working with an external resistance of 1000 Ω after three consecutive substrate loads and depending on the ion exchange membrane used: CMI (-··), AMI (–), and N-117 (··).

Figure 2. (a) Effect of current density on the power density depending on the type of membrane used: CMI (●), AMI (○), and N-117 (▼). (b) Maximum voltage (Vmax), maximum power density (Pmax), and internal resistance (Ωint) obtained for the three reactors through a polarization curve test.

Figure 3. (a) DGGE profiles on eubacterial *16S rRNA* amplified from liquor samples obtained in MFCs. (b) Principal Component Analysis (PCA) 2D-plot from digitalized DGGE profiles. DA: initial inoculum. 1, 2, and 3: electricity production peak number.

Figure 4. DGGE profiles on archaeal 16S rDNA amplified from samples obtained in MFCs. DA: initial inoculum. 1, 2, and 3: electricity production peak number.

Figure 5. (a) DGGE profile *16S rRNA* for the total archaea community. D.A: initial inoculums; Nafion-B: biofilm of the MFC equipped with Nafion N-117; Nafion-S: supernatant of the MFC (Nafion N-117); CMI-B: biofilm of the MFC (Ultrex CMI-7000); CMI-S: supernatant of the MFC (Ultrex CMI-7000). AMI-B: biofilm of the MFC (Ultrex AMI-7000); AMI-S: supernatant of the MFC (Ultrex AMI-7000).

Figure 6. (a) DGGE profile *16S rRNA* for the total eubacteria community. Nafion-B: biofilm of the MFC equipped with Nafion N-117; Nafion-S: supernatant of the MFC (Nafion N-117); CMI-B: biofilm of the MFC (Ultrex CMI-7000); CMI-S: supernatant of the MFC (Ultrex CMI-7000). AMI-B: biofilm of the MFC (Ultrex AMI-7000); AMI-S: supernatant of the MFC (Ultrex AMI-7000).

Figure 7. qPCR results for eubacterial *16S rRNA* (in black) and *mcr*A (in grey) genes, for the initial inoculum and for the different MFCs equipped with the three tested membranes and ratio *mcr*A/*16S rRNA* gene. DA: initial inoculum; AMI-S: supernatant of the MFC (Ultrex AMI-7000); AMI-B: biofilm of the MFC (Ultrex AMI-7000); CMI-S: supernatant of the MFC (Ultrex CMI-7000); CMI-B: biofilm of the MFC (Ultrex CMI-7000); Nafion-S: supernatant of the MFC (Nafion N-117); Nafion-B: biofilm of the MFC (Nafion N-117).