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**Start-up of electromethanogenic microbial electrolysis cells  
with two different biomass inocula for biogas upgrading**

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# Start-up of electromethanogenic microbial electrolysis cells with two different biomass inocula for biogas upgrading

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ABSTRACT: The performance and biomass enrichment of the biocathode of a pair of lab-scale two-chambered microbial electrolysis cells (MEC) were assessed for 95 days as a technology for upgrading the biogas produced in anaerobic digesters, converting CO<sub>2</sub> into CH<sub>4</sub> through the electromethanogenic process. Two different inocula were compared: i) a mixture of biomass from the anode of a MEC and anaerobic granular sludge (BC1); ii) biomass enriched in a methanol-fed upflow anaerobic sludge blanket reactor (UASB) (BC2). Quantitative and qualitative microbial community assessment of the enrichment process on the biocathodes was performed by means of high throughput sequencing of *16S rDNA* and *16S rRNA* based massive libraries, as well as RT-qPCR of *16S rRNA* and *mcrA* genes. Although BC2 had a faster increase in current density than BC1, there were no significant differences neither in the average CH<sub>4</sub> production (0.23±0.01 and 0.22±0.05 L m<sup>-3</sup> d<sup>-1</sup> for BC1 and BC2, respectively), nor in the

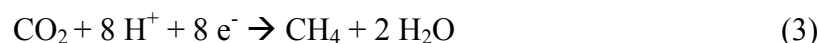
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3 cathodic methane recovery efficiency ( $65\pm 8$  and  $79\pm 17\%$ , respectively). Independently from the  
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5 origin of the inoculum, total and active archaeal microbial community in both biocathodes was  
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7 dominated by hydrogenotrophic methanogenic archaea, especially belonging to  
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9  
10 *Methanobacteriaceae* family (mainly *Methanobrevibacter* genus) ( $84-98\%$  of both *16S rDNA*  
11  
12 and *16S rRNA* relative abundance).  
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17 KEYWORDS: Electromethanogenesis, biocathode, hydrogenotrophic methanogen, biogas  
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19 upgrading, gene expression, RNA/cDNA.  
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## 22 INTRODUCTION

23  
24 Biogas is a renewable energy carrier gas consisting mainly of methane ( $\text{CH}_4$ , 40-75%) and  
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26 carbon dioxide ( $\text{CO}_2$ , 15-60%)<sup>1</sup> that is obtained from the anaerobic digestion of bio-degradable  
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28 materials such as manure, energy crops, household and industry wastes. So far, heating and  
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30 electricity generation are the main applications of biogas, which are spreading its use as an  
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32 alternative to fossil fuels. Moreover, it has more efficient uses, such as the injection in the  
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34 existent natural gas grid or the utilization as transport fuel. For the latter purposes raw biogas  
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36 needs some treatments prior to its use intended to remove undesired compounds (cleaning) and  
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38 adjust the calorific value separating  $\text{CH}_4$  from  $\text{CO}_2$  (upgrading), obtaining biomethane.  
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40 Conventional techniques for biogas upgrading, that are focused on  $\text{CO}_2$  removal without  
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42 changing  $\text{CH}_4$  mass, include pressure swing adsorption, membrane separation or chemical  $\text{CO}_2$ -  
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44 absorption, obtaining a final product with 95-97%  $\text{CH}_4$  and 1-3%  $\text{CO}_2$ .<sup>1</sup> An alternative to these  
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46 enrichment techniques that has recently emerged is the use of microbial electrolysis cells (MEC),  
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48 in which external energy is supplied to promote a thermodynamic no spontaneous reaction –such  
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50 as the bioelectrochemical  $\text{CO}_2$  conversion into methane in a process known as  
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3 electromethanogenesis.<sup>2-4</sup> This way, the methane yield from anaerobic digestion could be  
4 increased.<sup>5, 6</sup> The key players of the electromethanogenesis process are hydrogenotrophic  
5 methanogenic archaea that develop in the cathode compartment of the MEC (biocathode).  
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10 Previous studies have demonstrated that methane obtaining from CO<sub>2</sub> can be achieved through  
11 two different mechanisms of extracellular electron transfer, i) indirectly, through the  
12 intermediate abiotic electrochemical and/or microbially catalyzed production of hydrogen in the  
13 cathodic compartment (Equation 1 and 2); or ii) directly, by taking the electrons from the  
14 cathode and using them to reduce CO<sub>2</sub> to methane (Equation 3).<sup>2-4</sup>  
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31 The cathode potential required to enhance the electromethanogenic process due to potential  
32 losses is usually in the range from -0.4 to -1.4 V (vs. the standard hydrogen electrode, SHE).<sup>7</sup> At  
33 more negative potentials also acetate may be produced simultaneously with CH<sub>4</sub> and H<sub>2</sub> in a  
34 microbial biocathode based on mixed cultures.<sup>8</sup> However, acetate has been coproduced with  
35 methane at a fixed cathode potential as low as -590 mV (vs, SHE) in another study.<sup>9</sup>  
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43 Obtaining an enriched biomass in hydrogenotrophic methanogenic archaea in order to be used  
44 as inoculum could accelerate the start up of the methane producing MECs and improve methane  
45 production rates, being CO<sub>2</sub>/H<sub>2</sub> gassing<sup>10, 11</sup> or cultivation in an electrochemical bioreactor<sup>8, 12</sup> the  
46 most common enrichment methods. A recently proposed alternative enrichment method was the  
47 utilization of a methanol-fed upflow anaerobic sludge blanket reactor (UASB), providing that  
48 hydrogenotrophic methanogenesis is one of the possible routes for methanol degradation, besides  
49 the predominant methylotrophic route.<sup>13</sup> The effectiveness of this latter method to increase the  
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3 performance of an electromethanogenic biocathode needs to be further evaluated. Furthermore, a  
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5 deep study of the biomass harbored by methanogenic biocathodes is needed, applying new  
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7 techniques such as simultaneous DNA and RNA extraction, quantification and high throughput  
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9 sequencing, in order to disclose which microorganisms are really active among the ones that  
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11 might be present in the biofilm.<sup>13</sup> Previous works have focused only in describing existing  
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13 microorganisms, without deciphering the active ones.<sup>14-16</sup>  
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17 The main aim of this study was to assess the performance and biomass enrichment of the  
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19 biocathode of a lab-scale MEC to convert CO<sub>2</sub> into CH<sub>4</sub> as a technology for upgrading the biogas  
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21 produced in anaerobic digesters, comparing two different inocula: i) a mixture of biomass from  
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23 the anode of a MEC and anaerobic granular sludge; ii) biomass enriched in a methanol-fed  
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25 upflow anaerobic sludge blanket reactor (UASB). The microbial enrichment on the biocathodes  
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27 was assessed in terms of composition and activity using quantitative real-time polymerase chain  
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29 reaction (qPCR) and high throughput sequencing of 16S rDNA and 16S rRNA massive libraries.  
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### 33 MATERIALS AND METHODS

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35 **Experimental set-up.** A pair of identical two-chambered cells (0.5 L each compartment), constructed  
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37 using methacrylate, were operated (Figure 1). Both compartments were separated by a cation exchange  
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39 membrane (CEM) (dimensions: 14 x 12 cm; Ultrex CMI-7000, Membranes International Inc., Ringwood,  
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41 NJ, USA). Each chamber was filled with granular graphite with diameter ranging from 1 to 5 mm (Typ  
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43 00514, enViro-cell Umwelttechnik GmbH, Oberursel, Germany) to act as electrodes (anode and cathode),  
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45 remaining 265 mL of net volume in each compartment. Prior to being used, in order to remove metals and  
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47 organic residues, granular graphite was sequentially submerged in HCl (37%) and NaOH (1M) each for  
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49 24 hours, and then rinsed in deionized water and dried at 100 °C.<sup>17</sup> An A304 stainless steel mesh was used  
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51 as electron collector in each compartment (dimensions: 14 x 12 cm; mesh width: 6 x 6 mm; wire  
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53 thickness: 1 mm; Feval Filtros, S.L., Barcelona, Spain). An Ag/AgCl reference electrode (Bioanalytical  
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3 Systems, Inc., USA, +197 mV vs. standard hydrogen electrode (SHE)) was inserted in the cathode  
4 (working electrode) compartment, being the anode the counter electrode. A potentiostat (VSP, Bio-Logic,  
5 Grenoble, France) was used for data monitoring and poisoning the cathode potential in a three electrode  
6 mode. All potential values in this paper are referred to SHE. Anode and cathode potentials, and current  
7 were recorded every 5 min using a personal computer with EC-Lab software (Bio-Logic, Grenoble,  
8 France). The synthetic feeding solutions of the anode and cathode compartments, differing in the carbon  
9 source, contained (per liter of deionized water):  $\text{NH}_4\text{Cl}$ , 0.87 g;  $\text{CaCl}_2$ , 14.7 mg;  $\text{KH}_2\text{PO}_4$ , 3 g;  $\text{Na}_2\text{HPO}_4$ , 6  
10 g;  $\text{MgSO}_4$ , 0.246 g; and 1 mL  $\text{L}^{-1}$  of a trace elements solution. Additionally 2.9 g  $\text{L}^{-1}$  of  $\text{CH}_3\text{COONa}$  and  
11 5 g  $\text{L}^{-1}$  of  $\text{NaHCO}_3$  (since  $\text{CO}_2$  is mainly present as  $\text{HCO}_3^-$  at pH 7) were added at the anode and cathode  
12 solutions, respectively, as carbon source. The solution of trace mineral contained (per liter of deionized  
13 water):  $\text{FeCl}_3 \cdot \text{H}_2\text{O}$ , 1.50 g;  $\text{H}_3\text{BO}_3$ , 0.15 g;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.03 g; KI, 0.18 g;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.12 g;  
14  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.06 g;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.12 g;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.15 g;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.023 g; EDTA, 10 g.  
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29 **Reactor operation.** The MECs were operated in continuous for 95 days poisoning the cathode  
30 potential at -800 mV vs SHE. One of the MECs (BC1) was inoculated, both the anode and the  
31 cathode compartment, with 30 mL of a mixture 3.6:1 of the biomass of the anode of a mother  
32 MEC treating digested pig slurry<sup>18</sup> and granular biomass from a full-scale AD, with a volatile  
33 suspended solids (VSS) content of 16 g  $\text{L}^{-1}$ . The same mixture was used to inoculate the anode of  
34 the second MEC (BC2). In this case, the cathode was inoculated with 30 mL of a resuspension  
35 (VSS content of 33 g  $\text{L}^{-1}$ ) of the anaerobic granular sludge of an UASB that had been operated  
36 with methanol in order to enrich the biomass with methanogenic archaea.<sup>13</sup> The resuspension  
37 was done by vortex mixing during 10 minutes in a 50 mL tube containing 30 g of granular sludge  
38 and 25 mL of Ringer 1/4 sterilized solution. The influent solutions of both the anode and the  
39 cathode compartments were fed in continuous with a pump at 40 mL  $\text{h}^{-1}$  and recirculated with an  
40 external pump. The hydraulic retention time (HRT) of each compartment was of 6.8 h (with  
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respect to the net volume of each compartment), and the organic loading rate (OLR) of the anode compartment was established at  $7.83 \text{ kg}_{\text{COD}} \text{ m}^{-3} \text{ day}^{-1}$ . The MECs were operated at room temperature during the entire assay ( $23 \pm 2 \text{ }^\circ\text{C}$ ).

**Analytical methods and calculations.** Samples of the effluent of each compartment were analyzed for pH using a CRISON 2000 pH electrode, besides chemical oxygen demand (COD) in the anode compartment samples, according to Standard Methods 5220.<sup>19</sup> Methane was measured (through the determination of dissolved methane) in the cathode samples according to Henry's Law and the following method.<sup>20</sup> Around 2 mL catholyte samples were collected with a 5 mL syringe and injected with a needle in a 4 mL vacutainer. The vacutainers were shaken vigorously for 30 s and then allowed to stand for 1 h. Headspace gas was analyzed for  $\text{CH}_4$  using a gas chromatograph (CP-3800, Varian, USA). Dissolved  $\text{CH}_4$  was computed using the equation:

$$X_L = \frac{C_{\text{CH}_4} \cdot MV_{\text{CH}_4} \cdot MW_{\text{CH}_4} \cdot (V_T - V_L + \alpha V_L) \cdot 1000}{V_L} \quad (4)$$

where  $X_L$  is the concentration of  $\text{CH}_4$  ( $\text{mg L}^{-1}$ ) in the solution,  $C_{\text{CH}_4}$  is the concentration of  $\text{CH}_4$  (%) in the headspace 1 h after shaking,  $MV_{\text{CH}_4}$  is the molar volume of  $\text{CH}_4$  at  $25 \text{ }^\circ\text{C}$  ( $0.041 \text{ mol L}^{-1}$ ),  $MW_{\text{CH}_4}$  is the molecular weight of  $\text{CH}_4$  ( $16 \text{ g mol}^{-1}$ ),  $V_T$  is the volume (mL) of the vacutainer,  $V_L$  is the volume (mL) of the solution, and  $\alpha$  is the water:air partition coefficient at  $25 \text{ }^\circ\text{C}$  (0.03). Methane production was normalized to the net volume of the cathode compartment (0.265 L).

COD removal efficiency in the MECs was calculated based on the difference between anode influent and effluent concentrations divided by the influent concentration.

**Electrochemical measurements and calculations.** The current density ( $\text{A m}^{-3}$ ) of the MECs was calculated as the quotient between the current recorded by the potentiostat (A) and the net volume of the anode ( $\text{m}^3$ ).

The Coulombic efficiency (CE), or the fraction of electrons obtained from the consumption of COD at the anode compartment that are available for methane production at the cathode (Equation 5), the energy efficiency relative to electrical input recovered as methane ( $EE_e$ , Equation 6), the energy efficiency relative to the energy content of the substrate ( $EE_s$ , Equation 7) and the energy efficiency with respect to the energy input and the energy in the substrate ( $EE_{e+s}$ , Equation 8) were calculated as:

$$CE = \frac{M \int_0^t I dt}{F b q \Delta COD} \quad (5)$$

$$EE_e = \frac{n_{CH_4} \Delta G_{CH_4}}{\int_0^t I E_{ap} dt} \quad (6)$$

$$EE_s = \frac{n_{CH_4} \Delta G_{CH_4}}{n_S \Delta G_S} \quad (7)$$

$$EE_{e+s} = \frac{n_{CH_4} \Delta G_{CH_4}}{\int_0^t I E_{ap} dt + n_S \Delta G_S} \quad (8)$$

where  $M$  is the molecular weight of the final electron acceptor,  $I$  is the current (A),  $F$  is Faraday's constant ( $96485 \text{ C mol}^{-1}$ ),  $b$  is the number of electrons transferred per mole of  $O_2$ ,  $q$  is the volumetric influent flow rate ( $L d^{-1}$ ),  $\Delta COD$  is the difference in the influent and effluent COD,  $n_{CH_4}$  are the moles of produced methane,  $\Delta G_{CH_4}$  is the molar Gibbs free energy of  $CH_4$  oxidation by oxygen to carbon dioxide ( $-817.97 \text{ kJ mol}^{-1}$ ),  $E_{ap}$  is the applied voltage calculated as the difference between the cathode and anode potentials (V),  $n_S$  are the moles of acetate consumed and  $\Delta G_S$  is the molar Gibbs free energy of acetate oxidation to carbon dioxide ( $-844.61 \text{ kJ mol}^{-1}$ ).



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3 Finally, the cathodic methane recovery efficiency ( $R_{\text{cat}}$ ), defined as the fraction of electrons  
4 reaching the cathode that are recovered as methane, was calculated as:  
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$$R_{\text{cat}} = \frac{8 F n_{\text{CH}_4}}{\int_0^t I dt} \quad (9)$$

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11 Cyclic voltammeteries (CV) in turnover conditions, i.e. in the presence of substrate, were  
12 performed using a potentiostat (VSP, Bio-Logic, Grenoble, France) at the start (day 0) and the  
13 end (day 95) of the assays in each biocathode, in order to study the electroactive microbial  
14 biofilms developed on the cathodes. The same three-electrode configuration used for the MECs  
15 operation was maintained for the set up of the CV. The start ( $E_i$ ) and vertex ( $E_f$ ) potentials were -  
16 800 and 400 mV vs SHE, respectively, and the scan rate was set at 1 mV s<sup>-1</sup>.  
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28 **Microbial community analysis.** The bacterial communities in both inoculums used for the  
29 cathodes of BC1 and BC2 and the biofilm harbored in the same electrodes at the end of the assay  
30 were analyzed by culture-independent molecular techniques such as (RT) quantitative PCR (RT-  
31 qPCR) and high throughput sequencing (MiSeq, Illumina). To keep the nucleic acids stable,  
32 especially RNA, samples were stored at -80 °C.  
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40 **Nucleic acid extraction and cDNA synthesis.** Total DNA and RNA were extracted  
41 simultaneously in triplicate from known weights of each sample, which consisted on granular  
42 sludge for the inoculums and granular graphite collected from the cathode compartments for the  
43 biocathode biofilm. The PowerMicrobiome<sup>TM</sup> RNA Isolation Kit (MoBio Laboratories Inc.,  
44 Carlsbad, CA, USA) was used according to manufacturer's instructions, and cDNA was obtained  
45 from RNA following the protocol described elsewhere.<sup>13</sup> Throughout the paper, cDNA  
46 quantification (*16S rRNA* and *mcrA* genes) and 16S rRNA (cDNA) sequencing results refer to  
47 active microorganisms.  
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3 **Quantitative PCR assay (qPCR).** Gene copy numbers of eubacterial *16S rRNA* gene and  
4 *mcrA* gene (methanogenic archaeal methyl coenzyme-M reductase) were quantified by means of  
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6 quantitative real-time PCR (qPCR). Each sample was analyzed in triplicate by means of the three  
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8 independent DNA and RNA extracts. The analysis was carried out by using Brilliant II SYBR  
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10 Green qPCR Master Mix (Stratagene, La Jolla, CA, USA) in a Real-Time PCR System  
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12 Mx3000P (Stratagene) operated with the protocol described elsewhere.<sup>18</sup>  
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17 The standard curve parameters of the qPCRs were as follows (for *16S rRNA* and *mcrA*,  
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19 respectively): slope of -3.244 and -3.532; correlation coefficient of 0.998 and 0.999; efficiency  
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21 of 103 and 92%; showing that the reactions performed had a high efficiency.  
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24 **High throughput sequencing and data analysis.** The same DNA and RNA extracts from the  
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26 inocula and the biofilms from the biocathodes used for qPCR analysis were also used for 16S-  
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28 based high throughput sequencing purposes (MiSeq), following the specific steps described  
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30 elsewhere.<sup>18</sup> Bayesian Classifier database of the Ribosomal Database Group (RDP) was used to  
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32 taxonomically classify the obtained Operational Taxonomic Units (OTUs).<sup>21</sup>  
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36 The data obtained from sequencing datasets for eubacterial and archaeal populations were  
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38 submitted to the Sequence Read Archive of the National Center for Biotechnology Information  
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40 (NCBI) under the study accession number SRP072511.  
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44 Statistical correspondence analysis of MiSeq data was performed by means of XLSTAT 2014  
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46 software (Addinsoft, Paris, France). The diversity of the samples was evaluated with the number  
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48 of OTUs, the inverted Simpson index, Shannon index and Goods coverage using the Mothur  
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50 software v.1.34.4 (<http://www.mothur.org>),<sup>22</sup> normalizing all the estimators to the lowest number  
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52 of reads among the different samples.  
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## 55 **RESULTS AND DISCUSSION**

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3 **Operation performance.** The current densities produced by the BC1 and the BC2 during the  
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5 95 days of operation are shown in Figure S1a and S1b, respectively. The BC2 achieved current  
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7 densities between 150 and 200 A m<sup>-3</sup> after 5 days of operation, while the BC1 showed a more  
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9 progressive increase (the low current densities between days 8 and 14 are due to a MEC  
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11 destabilization after the performance of a cyclic voltammetry). However, the BC1 maintained a  
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13 current density between 100 and 150 A m<sup>-3</sup> from day 50 on, while the BC2 produced a current  
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15 density around 80 A m<sup>-3</sup>. Nevertheless, the average methane production was similar in both  
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17 MECs, 0.22-0.23 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> (Table 1). It is noteworthy that the obtained values are twenty-fold  
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19 and ten-fold higher, in terms of current density and methane production respectively than those  
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21 reported previously in a dual-chamber methanogenic MEC using graphite granules as electrodes,  
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23 with current density generation and methane production rate of 9.3 A m<sup>-3</sup> and 0.018 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>,  
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25 respectively;<sup>3</sup> they are also higher than those achieved from a spiral-wound-electrode MEC with  
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27 an applied voltage of 1.2 V, with a current density generation and methane production rate of  
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29 109 A m<sup>-3</sup> and 0.16 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>, respectively.<sup>12</sup> Even 0.28 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> were achieved in a MEC with  
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31 a semi-batch fed cathode.<sup>23</sup>

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39 Regarding the cathodic methane recovery efficiency (R<sub>cat</sub>) and energy efficiencies (EE<sub>e</sub>, EE<sub>s</sub>  
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41 and EE<sub>e+s</sub>), the values obtained for BC2 are higher than those of BC1, but due to the instability of  
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43 BC2 performance these values have a higher variability (Table 1). Previous works with a two-  
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45 chambered MEC using graphite granules as electrodes have achieved an energy efficiency  
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47 related to the electrical energy input (EE<sub>e</sub>) and of electrical input and substrate (EE<sub>e+s</sub>) of 57%  
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49 and 30%, respectively, in batch mode,<sup>3</sup> values very similar to the ones obtained in this study. On  
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51 the contrary, the CE achieved (33±10 for BC1 and 25±12 for BC2) are lower than previously  
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53 reported, such as 72-80%.<sup>24</sup> Possible sinks of electrons include methanogenesis, oxidation by  
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3 electron acceptors other than the anode, acetate conversion to microbial carbon storage  
4 molecules and bacterial growth.<sup>25</sup> The potential of the anode as electron acceptor determines the  
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6 bacterial yield through the energy gain per electron transferred.<sup>25</sup> In this study, anode potentials  
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8 were on average 0.05 V and 0.4 V in BC1 and BC2, respectively. The higher the anode potential,  
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10 the higher the energy gain for bacteria and growth. The high anode potential, particularly of  
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12 BC2, was probably promoting a higher loss of electrons on bacterial growth, resulting in a low  
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14 CE. Finally, the obtained  $R_{\text{cat}}$  ( $65 \pm 8$  for BC1 and  $79 \pm 17$  for BC2) are below the 96% previously  
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16 reported by Cheng et al.<sup>2</sup> or the 84-86% obtained by Zeppilli et al.,<sup>24</sup> but much higher than the  
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18 23.1% achieved by Van Eerten-Jansen et al.<sup>5</sup> or the  $24.2 \pm 4.7\%$  reported by Zhen et al.<sup>26</sup>  
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24 Figure S2 shows the cyclic voltammograms obtained in both biocathodes at the start (day 0)  
25 and the end (day 95) of their operation. The curves obtained at the start of the operation showed  
26 a low response to the different applied potentials, as a result of the recent inoculation. At the end  
27 of the assay the curves showed that the biofilm was established in both biocathodes, with a better  
28 performance of BC2 at potentials lower than -330 mV vs. SHE. However, the current densities  
29 obtained during the continuous operation of the MECs were lower than those achieved in the  
30 cyclic voltammetries, especially in BC2. Catalytic current for both biocathodes had an onset at  
31 approximately -300 mV, that could be related to the reduction of  $\text{CO}_2$  to methane ( $E' = -237$  to -  
32 303 mV for pH 7-8) or acetate ( $E' = -287$  to -352 mV for pH 7-8), in the case that this product  
33 was generated in the cathode compartment.<sup>27</sup> A second onset appeared at a potential near -800  
34 mV, which might be related to the hydrogen evolution reaction ( $2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$ ),<sup>28</sup> so it is  
35 possible that  $\text{H}_2$  was formed at the biocathode and immediately consumed by hydrogenotrophic  
36 methanogens.  
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**Evaluation of the electromethanogenic MEC as a biogas upgrading technology.** With the obtained results of methane conversion from CO<sub>2</sub> (0.23 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>), the performance of the electromethanogenic MEC as a biogas upgrading technology was assessed. Up to 0.58 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> of biogas (composition of 60% CH<sub>4</sub> and 40% CO<sub>2</sub>) could be treated in this biocathode to obtain near 100% CH<sub>4</sub>. This way, 1 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> of biogas could be treated to obtain a composition of 83% CH<sub>4</sub> and 17% CO<sub>2</sub>. When compared to the existing biogas upgrading technologies, that achieve a methane purity of 95-97%,<sup>1</sup> it has to be taken into account that these technologies remove CO<sub>2</sub>, without changing CH<sub>4</sub> mass, so the final volume of gas obtained is reduced. That is, for each m<sup>3</sup> of biogas treated on traditional biogas upgrading technologies, about 0.6 m<sup>3</sup> of CH<sub>4</sub> would be recovered, while in the biocathode the obtained volume would be of 0.9 m<sup>3</sup>. Since higher cathodic methane recovery efficiencies have been achieved in previous studies,<sup>2</sup> and the designs and materials for biocathodes are constantly improving,<sup>12</sup> a better performance for this new biogas upgrading technology can be expected in the future, so further research is needed focused on its scaling up.

#### **Microbial community assessment.**

**Quantitative evolution of cathode biomass.** qPCR results of the 4 samples, regarding DNA (present microorganisms) for both *16S* rRNA (eubacteria) and *mcrA* (methanogenic archaea) showed higher gene copy numbers in the final BC2 biofilm than in BC1, although of the same order of magnitude (Figure 2). However, when looking at cDNA (active microorganisms), methanogenic archaea in BC1 biofilm revealed themselves more active than in BC2, with more than one order of magnitude increase (2.95·10<sup>5</sup> and 9.86·10<sup>3</sup> *mcrA* transcript copy numbers g<sup>-1</sup>, respectively); while eubacteria 16S rRNA gene copy numbers in the BC2 biofilm were 3.2 times higher than in BC1. In spite of the quantitative differences of methanogenic archaea between

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3 both biocathodes, no significant differences were observed related to methane production, so  
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5 other mechanisms may be affecting the performance of the biomass. It could be possible that the  
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7 highest number of active eubacteria in the BC2 increased the synergies with archaea, which  
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9 could help to overcome their lower number in comparison with BC1, as will be discussed in the  
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11 following section.  
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15 **Sequencing results for eubacteria and archaea.** Table S1 shows the number of reads obtained for  
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17 the inoculums and the final biofilm of the biocathodes for eubacteria (3538 OTUS) and archaea  
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19 (725 OTUS). Figure 3a shows the relative abundance of eubacterial *phyla* for the four samples,  
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21 regarding DNA (present microorganisms) and cDNA (active microorganisms) forms. Both  
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23 inoculums had a different composition, with a higher relative predominance of *Proteobacteria*  
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25 (57%) and *Bacteroidetes* (61%) in the inoculums of BC1 and BC2, respectively, while at the end  
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27 of the assays the biofilms were enriched the opposite (45% for *Bacteroidetes* and 58% for  
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29 *Proteobacteria* in BC1 and BC2, respectively). The most active *phyla* in BC1 biofilm were,  
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31 according to cDNA results, *Bacteroidetes* (37%), *Firmicutes* (33%) and *Proteobacteria* (27%),  
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33 while *Proteobacteria phylum* (73%) was highly active in BC2 biofilm. A previous study also  
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35 found that *Proteobacteria* was the most predominant *phylum* in an electromethanogenic  
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37 biocathode (54% of the clones in the library).<sup>29</sup> At family level (Figure 3b), *Cyclobacteriaceae*  
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39 was the predominant one in the BC1 biofilm (30%), in spite of being *Pseudomonadaceae* the  
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41 most abundant family in the inoculum (46%). In the BC2 biofilm four families accounted for the  
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43 same relative abundance (11%): *Desulfovibrionaceae*, *Cyclobacteriaceae*, *Pseudomonadaceae*  
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45 and *Rhodocyclaceae*, while they had a low relative abundance in the inoculum (below 3% for  
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47 *Desulfovibrionaceae* and below 0.25% for the three other families). When looking at the cDNA  
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49 form, and thereby active microorganisms, *Cyclobacteriaceae* family maintained its higher  
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3 relative abundance (25%), accompanied by *Eubacteriaceae* (13%) in the BC1 biofilm. However,  
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5 the most active families in the BC2 biofilm where *Desulfobacteraceae* and *Desulfovibrionaceae*  
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7 (22 and 20%, respectively), although being practically inactive in the inoculum. Therefore, these  
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9 results suggest on the one hand that the active groups of microorganisms differ from the most  
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11 abundant ones; and on the second hand, the enrichment of eubacteria on the electromethanogenic  
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13 biocathodes under the same operational conditions may be different, which is consistent with a  
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15 recent work.<sup>15</sup>  
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21 *Pseudomonas* was one of the genera identified in the biocathodes with a higher relative  
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23 abundance (2 and 7% of active microorganisms in BC1 and BC2, respectively). Certain species,  
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25 such as *Pseudomonas aeruginosa*, have been described in microbial fuel cell (MFC) anodes, and  
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27 are able to potentially produce several shuttling compounds that facilitate electrochemical  
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29 activity.<sup>30</sup> The clear enrichment of OTUs belonging to this genus after inoculation suggest a role  
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31 in the biocathode processes. A similar enrichment was observed for *Geobacter*, a well known  
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33 exoelectrogenic eubacteria, which represented 3 and 4% of active microorganisms in BC1 and  
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35 BC2, respectively. *Geobacter* is able to catalyze bioelectrochemical hydrogen production at the  
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37 cathode,<sup>31, 32</sup> and may be involved in the production of methane through the microbially-  
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39 catalyzed production of H<sub>2</sub> (equation 1 and 2). Other remarkable genus because of its relative  
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41 abundance was *Desulfovibrio* (7 and 20% of active microorganisms in BC1 and BC2,  
42  
43 respectively), belonging to *Desulfovibrionaceae* family. Sulfate reducing species such as  
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45 *Desulfovibrio* have been reported involved in sulfide-mediated electron transfer in anodes.<sup>33</sup>  
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47 Since the latter family is more abundant in BC2, it could be the reason for its similar methane  
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49 production to BC1 in spite of the lower copy number of transcripts of *mcrA* gene obtained by  
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51 RT-qPCR. Furthermore, *Desulfovibrio* may also be involved in the production of methane  
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3 through the microbially-catalyzed production of H<sub>2</sub>, as described above for *Geobacter*. Finally,  
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5 *Acetobacterium*, belonging to *Eubacteriaceae* family, which has been identified previously in  
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7 methanogenic cathodes,<sup>4, 14, 15</sup> was particularly enriched in BC1 (11 and 0.1% of active  
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9 microorganisms in BC1 and BC2, respectively). *Acetobacterium* is a typical cathodic acetogen,<sup>9</sup>  
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11 and its presence could indicate that electrons were not exclusively directed to methanogenesis,  
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13 explaining the cathodic methane recovery below 100% obtained in both biocathodes. The  
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15 cathodic methane recovery was slightly lower in BC1 than in BC2 (65±8 and 79±17,  
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17 respectively), which also presented the highest relative abundance for *Acetobacterium*. This  
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19 hypothesis is reinforced by the fact that small amounts of acetate were detected in the cathode  
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21 effluent (< 33 mg L<sup>-1</sup>). Therefore, the role of the described eubacteria genera may reinforce and  
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23 countervail lower number of methanogenic population in BC2 and explain the similar methane  
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25 production in both biocathodes.  
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33 Regarding archaea population, Figure 4 shows a clear enrichment in *Methanobacteriaceae*  
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35 family in both biocathodes, especially in BC2 (90% of relative abundance), belonging mainly to  
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37 *Methanobrevibacter* genus. Apart from its high relative abundance, it also revealed as the most  
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39 active family (87 and 98% in BC1 and BC2, respectively). Previous work also determined that  
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41 *Methanobacteriaceae* was the predominant family on methanogenic biocathodes, although  
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43 identifying *Methanobacterium* as the predominant species,<sup>4, 6, 26</sup> such as 86.7% using fluorescent  
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45 in situ hybridization (FISH) in a two-chamber electrochemical reactor containing an abiotic  
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47 anode and a biocathode for methane production,<sup>2</sup> or > 93% of the total sequenced active archaeal  
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49 reads in a MEC with concomitant production of acetate, methane and hydrogen.<sup>9</sup>  
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51 *Methanobacterium* dominated also on the biocathodes, with lesser members of  
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53 *Methanobrevibacter*, in MECs inoculated with either anaerobic bog sediment where  
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3 hydrogenotrophic methanogens were detected or anaerobic digestion sludge dominated by the  
4 acetoclastic *Methanosaeta*.<sup>14</sup> Instead, *Methanobrevibacter* was found to dominate the biofilms  
5 developed on platinum cathodes (81-100%), while *Methanobacterium* abounded on the other  
6 cathode materials assayed (median of 97% in abundance of all archaea), when the inoculum used  
7 contained primarily the genus *Methanosaeta* (95%).<sup>15</sup> *Methanobacterium*, *Methanobrevibacter*  
8 and *Methanocorpusculum* dominated the biocathode of another MEC at an applied voltage of 0.7  
9 V,<sup>10</sup> and *Methanobrevibacter* and *Methanosarcina* were observed in a MEC with a cathode of  
10 graphite granules.<sup>24</sup> Therefore, regardless of the initial composition of the inoculums used, in this  
11 study, a convergent enrichment towards hydrogenotrophic methanogenic families was clear,  
12 especially in the case of the inoculum of BC2, which was initially enriched in methylotrophic  
13 methanogenic archaea (*Methanomassiliicoccaceae*,<sup>34</sup> 24%, and *Methanosarcinaceae*, 50%,  
14 genus *Methanomethylovorans* and *Methanolobus*<sup>35, 36</sup>). *Methanobrevibacter* genus, along with  
15 *Methanobacterium* found in other studies, seem to be especially adapted for growth in  
16 electromethanogenic MECs, as stated in a previous work,<sup>15</sup> differentiating from other  
17 hydrogenotrophic methanogens under poised potentials. Recently, it has been stated that the  
18 predominance of these genera may be due to the fact that they do not require complex carbon  
19 sources and growth factors such as vitamins and amino acids, while other hydrogenotrophic  
20 species are known to require acetate as a carbon source or peptone as a growth factor.<sup>37</sup>  
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48 **Biodiversity analysis.** Table S1 shows the results for the biodiversity analysis performed on  
49 inoculums and final biocathode biofilm samples. The Inverted Simpson and Shannon indexes for  
50 eubacteria and archaea population showed that in general biodiversity decreased when looking at  
51 the really active population in biofilms with respect to the present one. Eubacteria population in  
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3 the final biofilm of BC2 was the richest, both in presence and in activity according to the  
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5 Inverted Simpson index (16.36 and 9.90, respectively), while archaea population was richer in  
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7 the final biofilm of BC1 (2.46 and 2.30 for DNA and cDNA, respectively). These results agree  
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9 with the high relative abundance of *Methanobacteriaceae* in BC2 found in the MiSeq 16S-based  
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11 sequencing analysis, which reduces its biodiversity. On the other hand, the final biocathode  
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13 biofilms showed a lower biodiversity compared to the inoculums regarding archaea community,  
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15 as a result of their high enrichment in *Methanobrevibacter*. Eubacteria increased its biodiversity  
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17 in BC2 compared to the inoculum, which disagree with the results of a previous work that found  
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19 the opposite behavior.<sup>15</sup>  
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24 **Correspondence analysis.** Correspondence analysis results for eubacteria and archaea  
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26 community are shown in Figure S3a and S3b. The evolution from the inoculums to the final  
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28 biocathode biofilms was similar for both populations. Results show a clear differentiation  
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30 between BC1 and BC2 inoculums but, in spite of their diverse initial composition, their  
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32 populations evolved on the biocathodes towards consortiums that were clearly clustered together  
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34 at the end of the assay, as a clear example of convergent microbial enrichment. Furthermore,  
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36 DNA and cDNA for each sample were prochain, indicating that the active populations were  
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38 similar to the existing ones, in spite of the differences detected by the MiSeq sequencing. These  
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40 results corroborate that a very specific archaea population was obtained under the strict operation  
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42 conditions of both biocathodes, and that the different inocula used had little influence on the final  
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44 composition and activity, as was also suggested by the results obtained regarding methane  
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46 production and operation performance.  
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3 Results showed that the origin of the biomass tested in this study had little influence on the  
4 performance of the biocathode, since methane productions and energy efficiencies were similar  
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8 in two cells with different inoculum composition. Both inoculums converged at the end of the  
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11 MECs operation in a similar composition, especially for the archaeal communities, highly  
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13 dominated by hydrogenotrophic methanogenic archaea. *Methanobrevibacter* revealed itself as  
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15 the most active family in the cathode biofilm, according to the RNA-based high throughput  
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17 sequencing. Further research is needed to make electromethanogenic biocathode MEC (based on  
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19 CO<sub>2</sub> conversion into CH<sub>4</sub>) a promising technology for biogas upgrading, able to compete in the  
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21 future with the existing technology (based on CO<sub>2</sub> removal).  
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## 26 27 **ASSOCIATED CONTENT**

28  
29 Table S1: diversity indexes  
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33 Figures S1-S3: S1 (current density profiles); S2 (cyclic voltammograms); S3 (correspondence  
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35 analysis).  
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### 49 50 **Notes**

51  
52 The authors declare no competing financial interest.  
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**Table 1.** Performance of BC1 and BC2 (average±standard deviation). Abbreviations: BC: biocathode; CE: coulombic efficiency; R<sub>cat</sub>:cathodic methane recovery; EE<sub>e</sub>: energy recovery related to electrical input; EE<sub>s</sub>: energy recovery related to substrate; EE<sub>e+s</sub>: energy recovery related to substrate and electrical input.

<b>Parameter</b>	<b>BC1</b>	<b>BC2</b>
CH <sub>4</sub> production (m <sup>3</sup> m <sup>-3</sup> d <sup>-1</sup> )	0.23±0.01	0.22±0.05
CE (%)	33±10	25±12
R <sub>cat</sub> (%)	65±8	79±17
EE <sub>e</sub> (%)	57±17	61±16
EE <sub>s</sub> (%)	23±6	54±34
EE <sub>e+s</sub> (%)	17±2	33±18

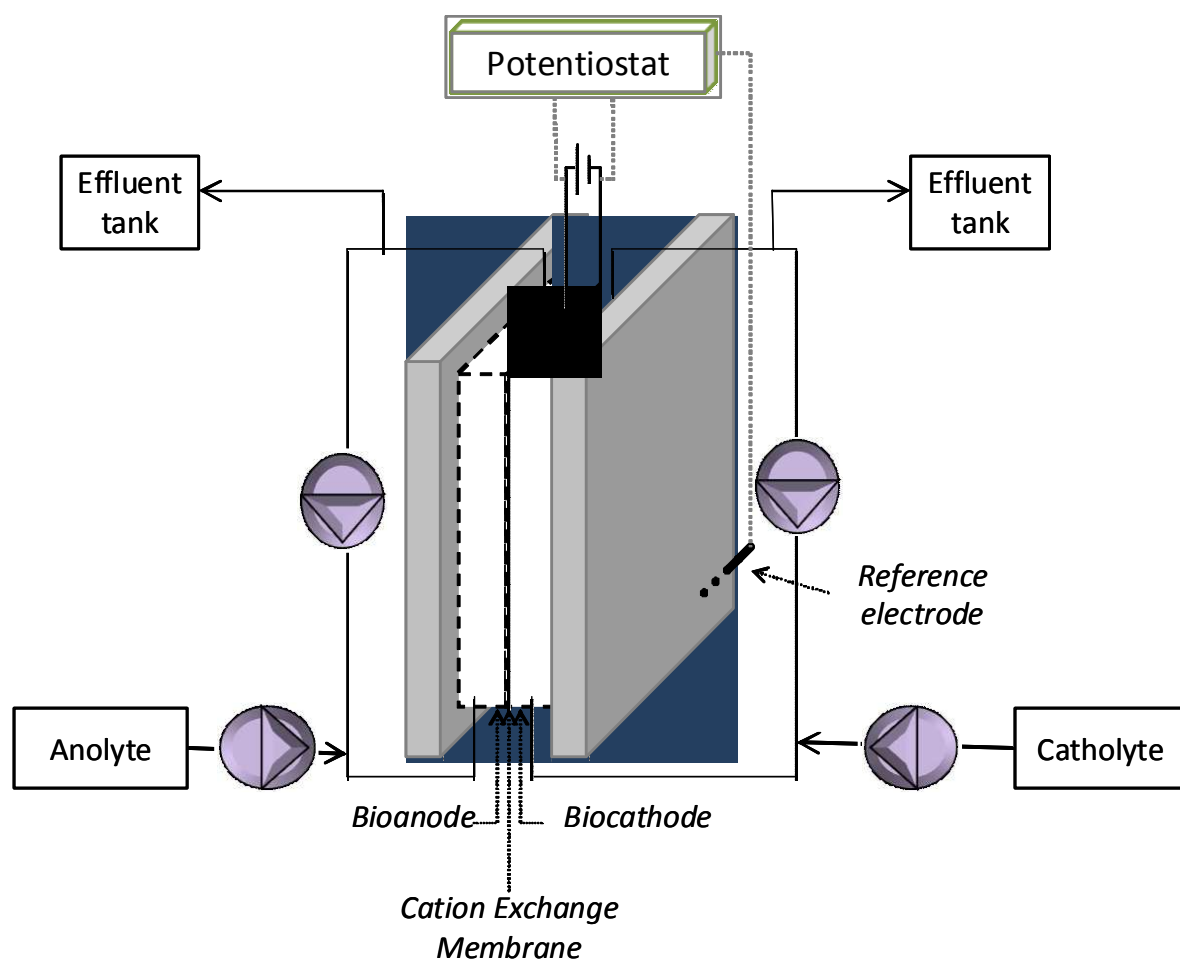
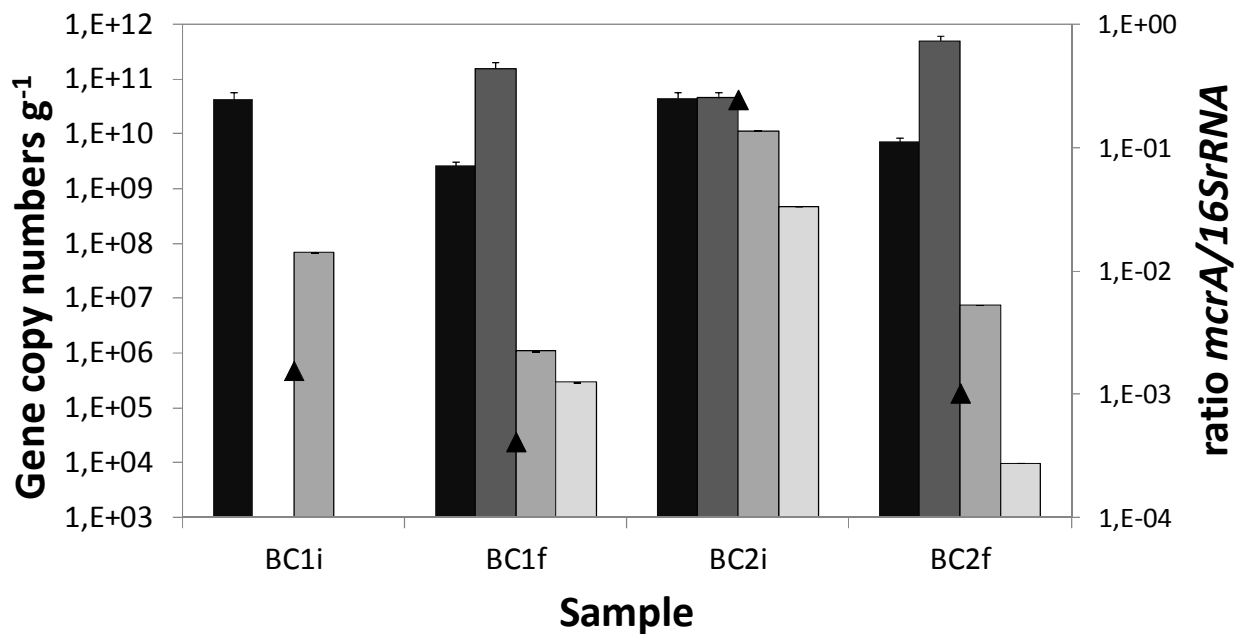
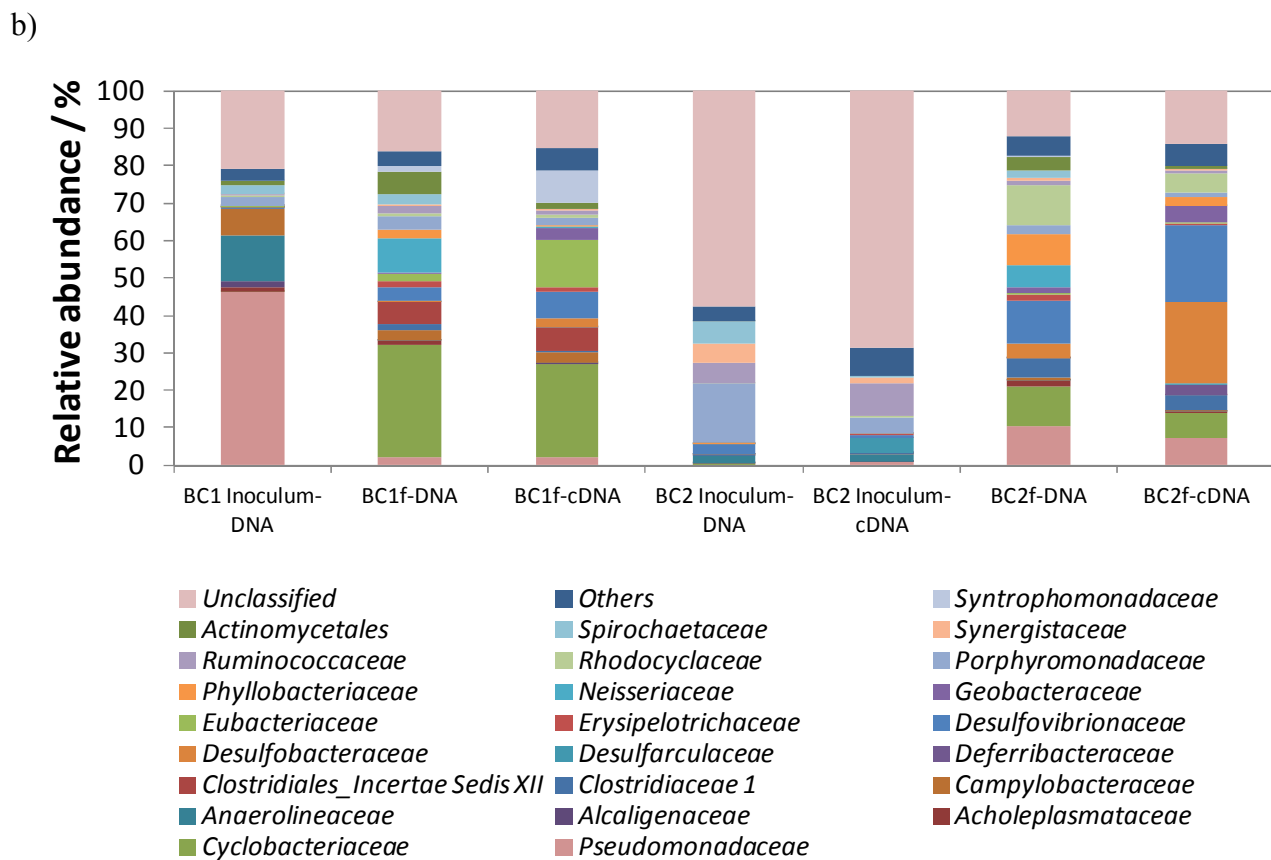
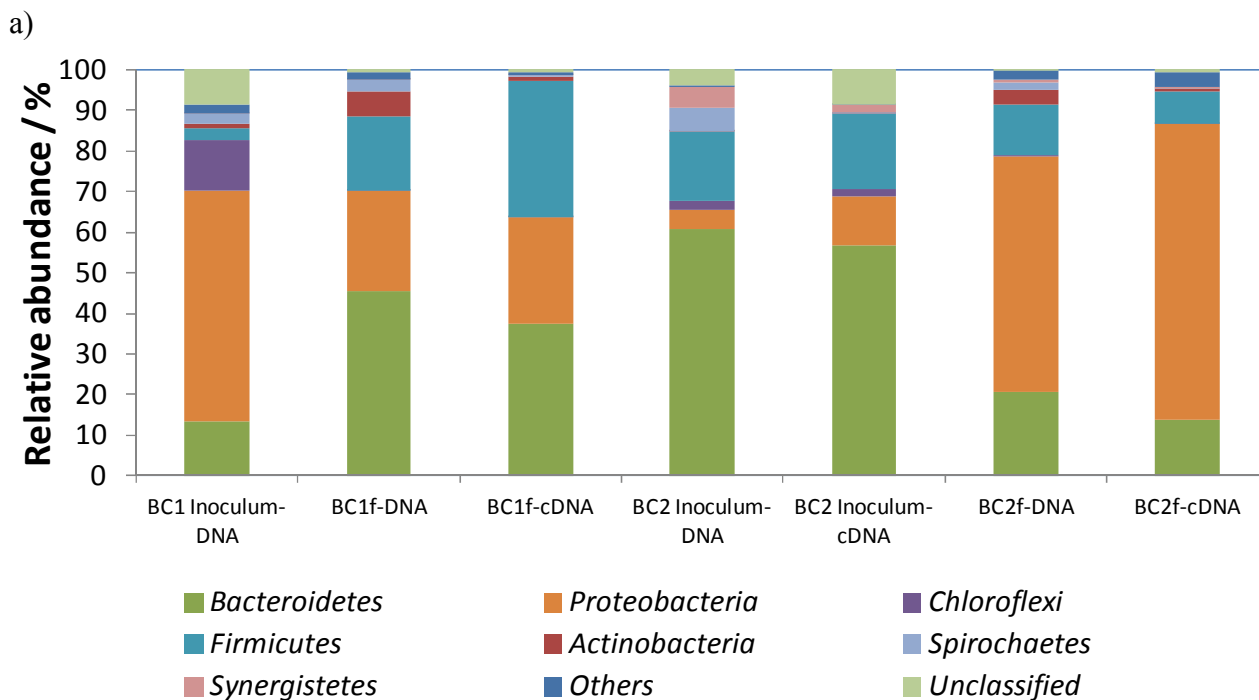


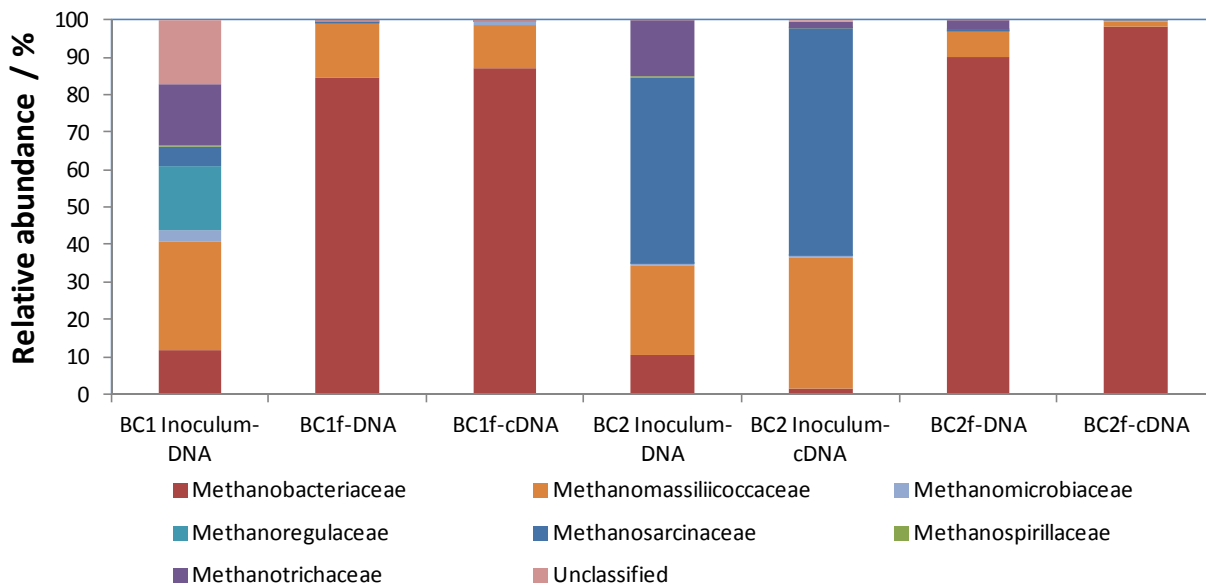
Figure 1. Scheme of the reactor set-up.



**Figure 2.** Gene copy numbers and transcripts of *16S* rRNA and *mcrA* genes from initial inoculum of BC1 and BC2 (i) and the final enrichment on each biocathode (f). *16S* rRNA-DNA, in black bars; *16S* rRNA-cDNA, in dark grey bars; *mcrA*-DNA, in medium grey bars; *mcrA*-cDNA, in light grey bars; ratio *mcrA/16S* rRNA, in triangle.



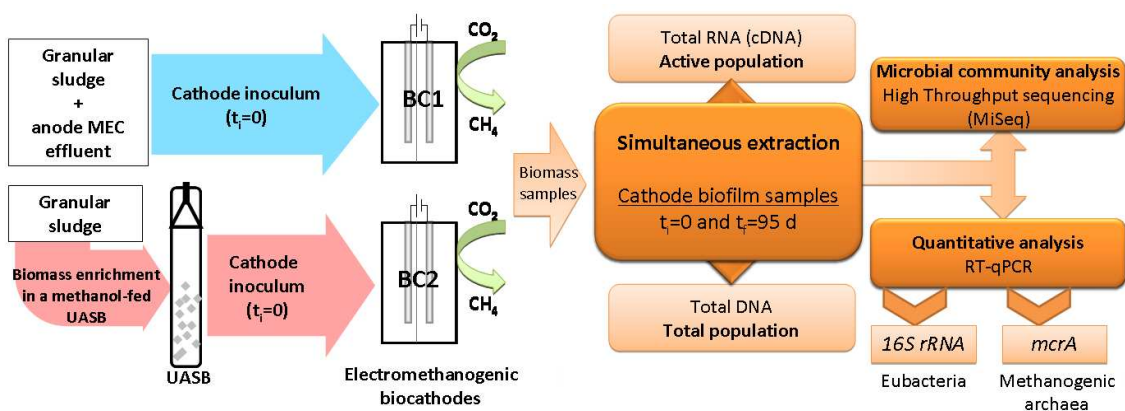
1  
2  
3 **Figure 3.** Taxonomic assignment of sequencing reads from Eubacterial community of the  
4 inoculum of BC1 and BC2 and the final sample of each cathode for DNA (total population) and  
5 cDNA (active populations), at a) phylum b) family levels. Relative abundance was defined as the  
6 number of reads (sequences) affiliated with any given taxon divided by the total number of reads  
7 per sample. Phylogenetic groups with a relative abundance lower than 1% were categorized as  
8 “others”.  
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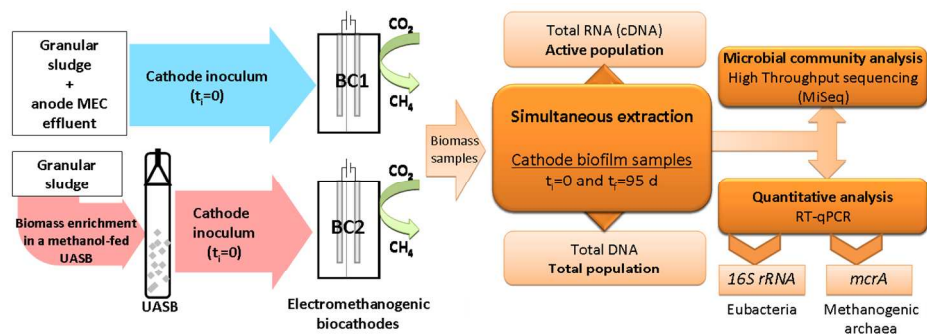
**Figure 4.** Taxonomic assignment of sequencing reads from Archaeal community of the inoculum of BC1 and BC2 and the final sample of each cathode for DNA (total population) and cDNA (active populations) at family level. Relative abundance was defined as the number of reads (sequences) affiliated with any given taxon divided by the total number of reads per sample. Phylogenetic groups with a relative abundance lower than 1% were categorized as “others”.



## TABLE OF CONTENT (TOC) GRAPHIC



SYNOPSIS. Microbial electrolysis cells (MEC) with electromethanogenic biocathodes were assessed as a technology for upgrading the biogas produced in anaerobic digesters.



Graphical abstract

394x141mm (96 x 96 DPI)