Impact of the start-up process on the microbial communities in biocathodes for electrosynthesis

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7

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22

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30

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Abstract

This study seeks to understand how the bacterial communities that develop on biocathodes are influenced by inocula diversity and electrode potential during start-up. Two different inocula are used: one from a highly diverse environment (river mud) and the other from a low diverse milieu (anaerobic digestion). In addition, both inocula were subjected to two different polarising voltages: oxidative (+0.2V vs. Ag/AgCl) and reductive (-0.8V vs. Ag/AgCl).

Bacterial communities were analysed by means of high throughput sequencing. Possible syntrophic interactions and competitions between archaea and eubacteria were described together with a discussion of their potential role in product formation and current production. The results confirmed that reductive potentials lead to an inconsistent start-up procedure regardless of the inoculum used. However, imposing oxidative potentials help to quickly develop an electroactive biofilm ready to withstand reductive potentials (i.e. biocathodic operation). The microbial structure that finally developed on them was highly dependent on the raw community present in the inoculum. Using a non-specialised inoculum resulted in a highly specialised biofilm, which was accompanied by an improved performance in terms of consumed current and product generation. Interestingly, a much more specialised inoculum promoted a rediversification in the biofilm, with a lower general cell performance.

Keywords: Bioelectrochemical systems, biocathode, microbial electrosynthesis, start-up, CO_2 reduction, high throughput sequencing

1. Introduction

Most of the carbon-based chemicals and fuels currently produced throughout the world are derived from non-renewable sources (i.e. fossil resources). They are the basic feedstock for many industrial processes and are present in most human activities. Yet, their production, transformation and utilisation are usually accompanied by the release of large amounts of CO_2 into the atmosphere. In an effort to limit the burden that these commodities place on the environment, innovative technologies and novel industrial processes have emerged in recent years, including CO_2 capture and utilization technologies [1,2]. This has given birth to the concept of biorefinery [3], a term that encompasses those industrial activities that integrate biomass conversion and the production of fuels, energy and value-added chemicals, such as methane, ethylene, ethylene-glycol or monomers for plastics like acrylic acid [2]. Processes that use CO_2 rich streams as a feedstock are of special interest, as this contributes to further reduce their environmental impact. Microbial electrosynthesis (MES) is a novel technology that can be framed within this group [4], although the ability of MES to use organic compounds (e.g. acetate, ethanol) as a substrate, also opens the possibility to upgrade organic feedstocks.

MES is based on the ability of certain strains of electroactive bacteria to directly or indirectly take electrons from a solid surface (usually termed as biocathode) and use them in their metabolism to produce chemicals such as carboxylic acids or combustible gases [5], depending on process design and conditions. For more information on the basics of MES, we refer the reader elsewhere [6–9].

MES opens a wide variety of possibilities to produce valuable organic compounds. The range of target products attainable is mainly restricted by the substrate (CO_2 or organic compounds) and the operational conditions (culture medium, pH, electrode potential and the microbial community present on the electrodes (mixed or pure culture biofilms) [6]). For instance, pure cultures of species like *Sporomusa Ovata* [10] or *Clostridium Ljungdahlii* [11] have been reported to be efficient at producing commodity chemicals from inorganic carbon on biocathodes. On the other hand, mixed cultures harvested from sediments, sludge or other natural environments have proven to be more robust when fed with real waste streams. Although mixed cultures provide lower efficiencies in product generation, they have a promising potential for practical applications [11,12] as they allow to operate in continuous conditions, can be fed with mixed (non-sterilized) substrates and display a better adaptive capacity [13]. Acetic acid is the most reported product from CO_2 bioelectroreduction; it is generated mainly following the Wood-

Ljungdahl pathway [14], requiring the presence of homoacetogens such as *Sporomusa sp.* and *Clostridium sp.* These species are commonly found in mixed culture biocathodes, and are responsible for the production not only of acetic acid, but also some other organic products from mixtures of CO_2 and H_2 [15]. All these products can be obtained alone or simultaneously in biocathodes, giving way to mixtures of carboxylic acids [16]. Moreover, biocathodes are also capable of performing chain elongation reactions, using short chain carboxylic acids as building blocks [17].

Laboratory scale MES is typically carried out in three-electrode two-chamber arrangements, and for the cathodic reaction to proceed, moderate potentials (usually in the range of -0.6 V and -1.1 V vs. Ag/AgCl) are required depending on the system overpotentials [18] and the target products. The minimum feasible threshold potential is limited by the hydrogen evolution reaction. Unfortunately, and contrary to what happens to bioanodes, the inoculation and start-up of biocathodes is usually an inconsistent, tedious and time-consuming procedure [11,19,20]. Biocathodes are usually started up directly in a cathodic mode of operation (i.e. by imposing cathodic potentials) [11,19], but they can also be started-up in an anodic mode of operation (i.e. by imposing anodic potentials) and then converted into biocathodes by reversing the potential to typical cathodic values [21–23].

The present study aims at gaining knowledge on how the start-up process influences the microbial communities that develop on the biofilm of biocathodes. This is done by assessing the impact of the microbial diversity of the inoculum and the starting potential of the bioelectrode. For this purpose, we tested a highly diverse inoculum such as river mud (RM), and a less species richness one such as anaerobic digestate (AD). We also evaluated the impact of the starting potential by either operating the working electrode as an anode and then switching it to cathode, or directly operating the working electrode as a cathode. This approach resulted in four different start-up strategies, and for all of them, we provide an analysis of the evolution of microbial communities together with information of the reactor performance (in terms of current production and product formation).

2. Materials and Methods

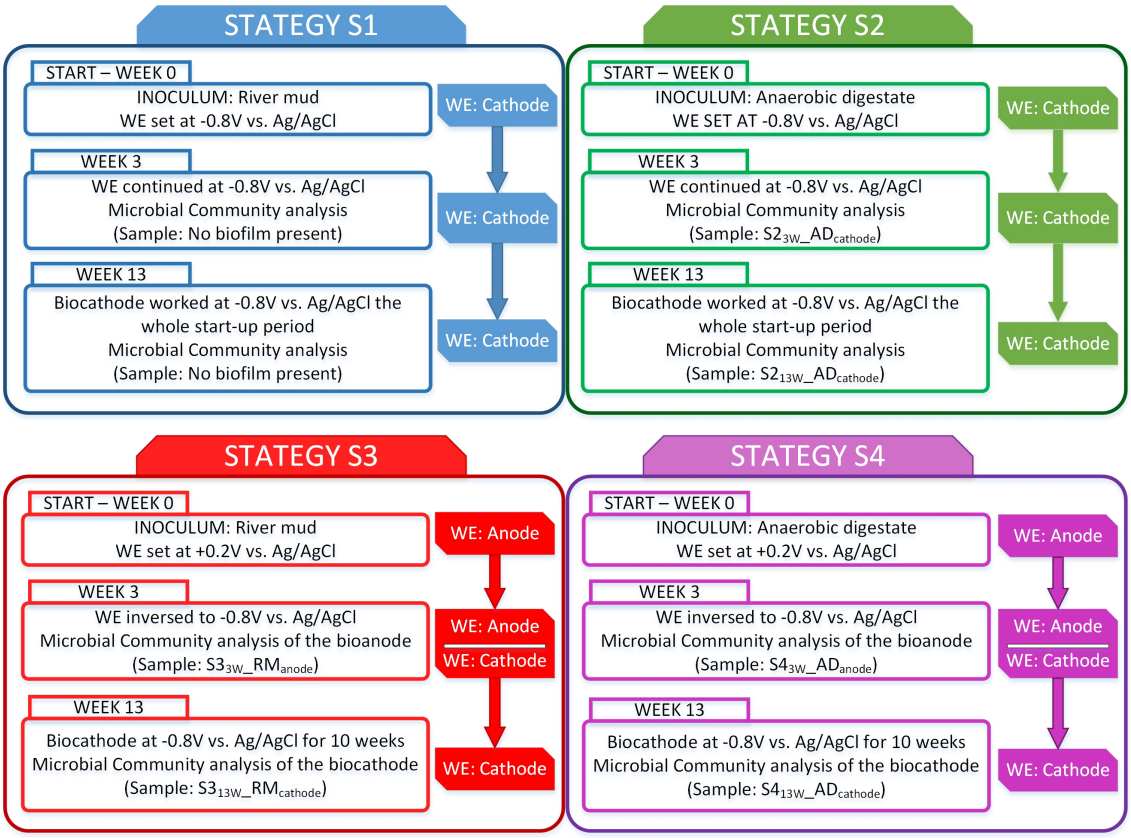
2.1 MES reactors set-up

Twelve identical two-chambered planar cells were constructed with polycarbonate plates, providing a working cathodic volume of 50 mL and 15 mL of headspace. A pretreated Nafion 117 (Cation Exchange Membrane (CEM)) was used to separate the

anodic and cathodic compartments. Both the working and counter electrodes (WE and CE, respectively) were made of carbon felt (SGL Group, Germany) due to the suitability of this material to work as cathode or anode, providing chemical stability in both cases. No specific current collectors were used in our set-up. To provide an intimate contact between the electrodes and titanium wire, it was sewed through the carbon felt. All assemblies provided a contact resistance < 2 ohm. Electrodes were pretreated by subsequent immersion in nitric acid 1M, acetone 1M and ethanol 1M during 24h each to avoid hydrophobicity and impurities. Then, the electrodes were rinsed in demineralised water to ensure absence of organics from the pretreatment. The electrodes and the membrane had a projected surface area of 19.6 cm^2. All cells worked on a three-electrode configuration using an Ag/AgCl reference electrode (0.20 vs. SHE; the stability of the reference electrode was checked prior to every batch cycle). The catholyte was continuously stirred using a magnetic stirrer at 200 rpm, and gas was collected from a built-in rubber septum.

2.2 Start-up strategies and operation

Four different start-up strategies (designated as **S1, S2, S3** and **S4**) were tested in triplicate, resulting in a total set of 12 cells (Schematic 1). Each strategy was characterised by the inoculum (**AD** or **RM**) and the start-up procedures (either operating the WE as an anode and then switching to a cathode or directly operating the WE as a cathode). Anodic potentials for three-electrodes configurations are usually in the range of -0.2V to +0.2V vs. Ag/AgCl. In our study, we selected a high potential (+0.2V vs. Ag/AgCl) to ensure favourable conditions for anodic biofilm formation. Cathodic potentials for three-electrodes configurations are usually in the range of -0.4V to -1.4V vs. Ag/AgCl. Again to favour a cathodic biofilm formation and to avoid extensive electrolytic hydrogen production, a relatively low cathodic potential was selected (-0.8V vs. Ag/AgCl).



Schematic 1. Strategies overview

For strategy **S1** the WE was started directly as a biocathode using RM as inoculum. The WE potential was set at -0.8 V vs. Ag/AgCl. Strategy **S2** was similar to S1 but using AD as inoculum.

In strategy S3, the WE was started as a bioanode and was inoculated with RM. The WE potential was set at +0.2 V vs. Ag/AgCl. After a period of 3 weeks (following the start-up), once the anodes of the working cells had developed a clear current response, the electrode potencial was poised at -0.8 V vs. Ag/AgCl to force them to operate as biocathodes. Strategy S4 was similar to S3 but with AD as inoculum.

Following the start-up, the cathodes were operated in batch mode. At the beginning of every batch cycle the WE and CE chamber were replenished with fresh culture medium/electrolyte. The duration of the batch cycles was fixed to 2 weeks to provide enough time for bacterial growth during the start-up period. The cells were maintained at 30 ºC and initial pH of the catholyte was 7.4.

2.3 Influents and inocula

The culture medium used for the WE chamber consisted of a synthetic nutrient solution with a composition (in $g \cdot L^{-1}$): 0.87 K_2HPO_4; 0.68 KH_2PO_4; 0.25 NH_4Cl; 0.1 KCl; 0.04

$CaCl_2 \cdot 2H_2O$; 0.45 $MgCl_2 \cdot 6H_2O$ and 10 ml per litre of a trace mineral solution containing (in $g \cdot L^{-1}$): 6 $MgSO_4 \cdot 7H_2O$, 1 $MnSO_4 \cdot H_2O$, 2 NaCl, 0.2 $FeSO_4 \cdot 7H_2O$, 0.3 $CoCl_2 \cdot 6H_2O$, 0.2 $CaCl_2 \cdot 2H_2O$, 0.17 $ZnCl_2$, 0.02 of $CuSO_4 \cdot 5H_2O$, 0.02 H_3BO_3, 0.04 $Na_2MoO_4 \cdot 2H_2O$, 0.06 $NiCl_2 \cdot 6H_2O$, 0.6 mg Na_2SeO_4 and 0.8 mg $Na_2WO_4 \cdot 2H_2O$ as described in [24]. When the WEs were operated as bioanodes, the carbon source consisted of a mixture of sodium acetate 0.5 $g \cdot L^{-1}$, sodium propionate 0.1 $g \cdot L^{-1}$ and glucose 0.1 $g \cdot L^{-1}$. When they operated as biocathodes, the carbon source was sodium bicarbonate 2.5 $g \cdot L^{-1}$. All nutrient solutions were prepared immediately before each batch cycle to avoid microbial pre-contamination. The electrolyte used in the CE chamber was a phosphate 0.1 M buffer solution. For CE operating as chemical anodes, the pH was slightly alkaline (7.8) to counteract their natural tendency towards acidification. For similar reasons, the pH of CE operating as chemical cathodes was slightly acidic (6.8). Chemicals and reagents used were of analytical grade, and distilled water was used for medium preparation.

Two different inocula were used in this study: river mud taken from the sediments of a local river (Porma River, Province of Leon, Spain), and anaerobic digestate (AD) taken from the effluent of an anaerobic digester operating in the local wastewater treatment facility (Leon city WWTP, 200.000 i.e.). These inocula were diluted in oxygen-free culture medium (20/80 v/v) before being fed to the WE chamber. The cells were inoculated within a period of 3-4 hours after the inocula were collected.

2.4 Measurements and analytical techniques

Liquid samples were collected from the cathodic and anodic chambers and analysed immediately afterwards. Gas samples were collected with a GASTIGHT 1001 (Hamilton Co., GR, Switzerland) syringe from a built-in rubber septum.

Total organic carbon (TOC) and total nitrogen (TN) content were measured using a thermocatalytic oxidation system Analytikjena Multi N/C_3100. Volatile fatty acids (VFAs) were analysed using a gas chromatograph (Varian CP3800 GC) equipped with a thermal conductivity detector and a Nukol capillary column (30 m × 0.25 mm × 0.25 μm) from Supelco, using He as mobile phase as described by [25] (detection limit 5 $mg \cdot L^{-1}$). Conductivity and pH were determined using APHA standard methodologies as described by [26]. Conductivity was determined with a HACH CDC401 probe in a Hach HQ40d multimeter, while pH was determined with a HACH 5014T probe in a CRISON 20+ pH meter. Electrochemical tests were performed using a potentiostat (VMP3, Biologic Science Instruments).

2.5 High throughput sequencing of massive 16S rRNA gene libraries

Samples from microbial community analysis were taken from both inocula used, **AD inoculum** and **RM inoculum**, and from the biofilms after 3 and 13 weeks of operation for each working strategy (as the culture medium is completely replaced after every batch cycle, the influence of immobilised biofilm communities is much more relevant than planktonic communities). It is important to clarify the terminology for the samples taken for the strategy S2 (**S2$_{3w}$_AD$_{cathode}$** and **S2$_{13w}$_AD$_{cathode}$**), strategy S3 (**S3$_{3w}$_RM$_{anode}$** and **S3$_{13w}$_RM$_{cathode}$**) and strategy S4 (**S4$_{3w}$_AD$_{anode}$** and **S4$_{13w}$_AD$_{cathode}$**). A thin piece of electrode (2mm x 2mm) was cut off with a stainless steel surgical blade in sterile conditions in a laminar flow cabinet and genomic DNA was extracted with the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's instructions. All PCR reactions were carried out in a Mastercycler (Eppendorf, Hamburg, Germany) and PCR samples were checked for size of the product on a 1% agarose gel. The PCR conditions are described in detail in section S1 of supplementary information.

The entire DNA extract was used for high throughput sequencing of *16S-rRNA* gene-based massive libraries (total eubacterial and archaeal). Each sample was amplified with *16S-rRNA* gene-based primers for eubacteria and archaea, respectively. The primer set used was 27Fmod (5`-AGRGTTTGATCMTGGCTCAG-3`) / 519R modBio (5`-GTNTTACNGCGGCKGCTG-3`) [27] and Arch 349F (5´-GYGCASCAGKCGMGAAW-3`) / Arch 806R (5`-GGACTACVSGGGTATCTAAT-3`) [28], respectively, for the eubacterial and archaeal analysis population. The obtained DNA reads were compiled in FASTq files for further bioinformatics processing and following the procedure described by [29]. Operational taxonomic units (OTUs) were then taxonomically classified using the Ribosomal Database Project (http://rdp.cme.msu.edu). Raw pyrosequencing data obtained from this analysis were deposited in the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) under nucleotide sequence accession numbers SRP115155, for eubacterial and archaeal population.

Microbial richness estimators (*observed OTUs* and *Chao1*) and diversity indices estimators (Shannon (*H')* and *1/Simpson*) were calculated using MOTHUR software, version 1.35.1, and normalizing the number of reads of all samples to those of the sample with the lowest number of reads. A heatmap for species abundance was completed using RStudio.

3. Results

3.1 Cell performance

The results of cell performance for every strategy are reported in this section. Current production and product formation were selected as performance indicators.

3.1.1 Current production

Important differences in the behaviour of the cells, in terms of current production, were observed depending on the start-up strategy. These differences are summarised in Table 1, and for more detailed information about temporary current profiles on every replicate, we refer the reader to Fig. S1 in supplementary information (section S2). For strategy S1, where the electrodes were operated at reductive potential (as cathodes) using RM as inoculum, no current production was observed in any of the three replicates.

Table 1: Maximum recorded currents for each strategy and lag periods observed.

Strategy	**Maximum current (A/m^2)**		**Comments**
	3 weeks	13 weeks	
S1 (RM:cathode-cathode)	<0.01	<0.01	No current or products.
S2 (AD:cathode-cathode)	0.4 ± 0.1	0.5 ± 0.1	Initial lag period of 2 weeks.
S3 (RM:anode-cathode)	0.6 ± 0.1	1.0 ± 0.2	Initial lag period of 24 h. Biocathodes took 4 days to produce current.
S4 (AD:anode-cathode)	0.7 ± 0.1	0.4 ± 0.1	Initial lag period of 24 h. Biocathodes took 3 days to produce current.

In strategy S2, the electrodes were also operated as cathodes but AD was used as inoculum. In this situation, and after a lag-phase of 2 weeks, the cells produced a stable current of 0.4 A/m^2, growing to 0.5 A/m^2 at the end of the experiment (13 weeks). For strategies S3 and S4 the electrodes were initially operated as anodes, using RM and AD, respectively, as inoculum. In both situations, an oxidative current was almost immediately produced (after a short lag-phase around 24 h; see Fig. S1, supplementary material, section S2) reaching moderate and stable peak values at the end of the 3-week interval. After this period, the bioanodes were switched into biocathodes by imposing a reductive potential. For strategy S3, this inversion resulted

in a lag-phase of 4 days, after which reduction currents started to grow steadily, stabilising at around 1 A/m^2 by the end of the test (week 13). The MECs in strategy S4 followed a similar pattern, although the currents at the end of week 13 were appreciably lower.

3.1.2 Product formation

Chemical production was measured at the end of every batch cycle and only acetic acid was detected in the electrolyte of all reactors (Fig. 1). Other volatile fatty acids (C2-C7) and alcohols (C1-C6) were not present above the detection limit of the chromatographs. Hydrogen was detected in the cathodic head-spaces in strategies S2, S3 and S4, while methane was detected only in strategy S2. However, total gas production could not be accurately quantified due to gas leakages detected in the set-up.

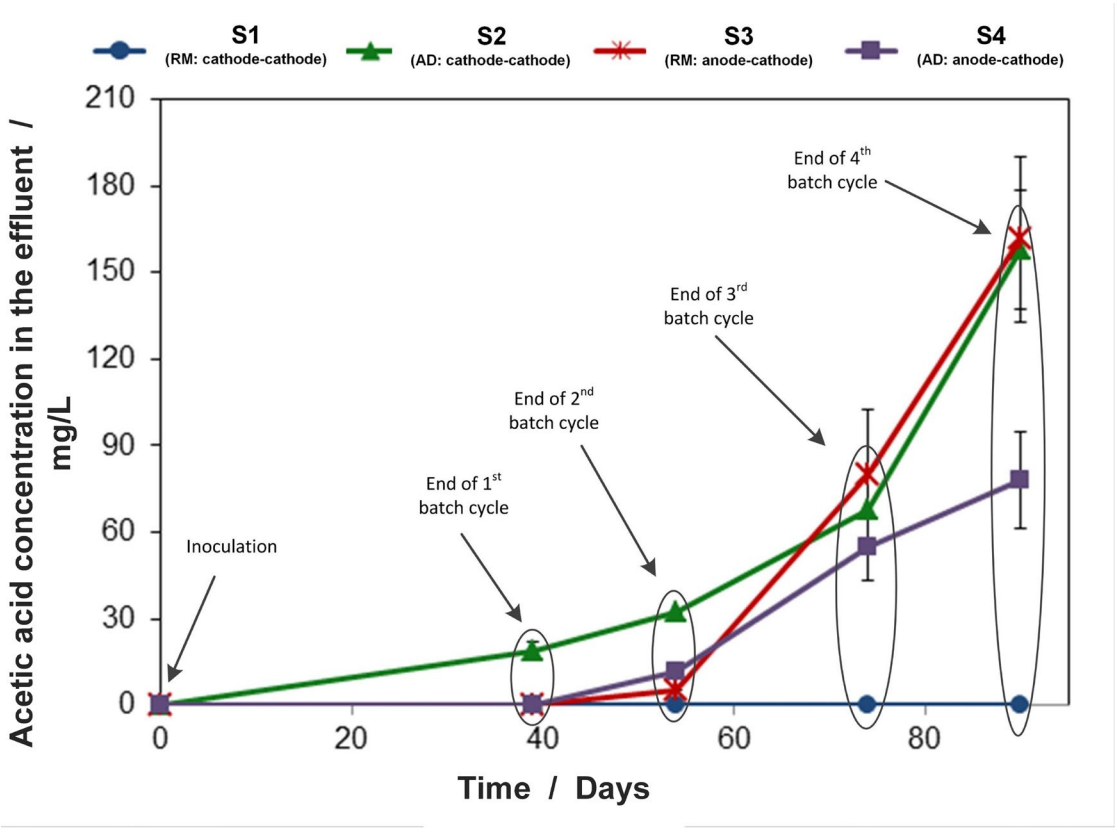


Fig. 1. Averaged acetic acid concentration at the end of every batch cycle and for each strategy (error bars show standard deviation for three replicates). Day 0 corresponds to inoculation.

The absence of any measurable current in strategy S1 resulted in no acetate production (Fig. 1). For S2, some acetic acid was found from the beginning of the

experiment, rising up to 158 mg/L at the end of the 13-week period. For S3, acetic acid production began to appear in small quantities at the end of week 7 after the first cathodic cycle, and rose sharply to 162 mg/L at the end of the final cycle. A similar behaviour was observed in S4, although the final acetate concentration was much lower (Fig. 1). Above all, Fig. 1 shows how titers consistently increase with every batch cycle for all strategies (except for S1), which might be indicative of a progressive acclimation and development of microbial communities.

Table 2: Cell performance for each start-up strategy

Strategy	Average current (A/m^2)	Present in Off-gas		Coulombic efficiency (%)
		H_2	CH_4	
S1 (RM:cathode-cathode)	0	No	No	n.a.
S2 (AD:cathode-cathode)	0.45	Yes	Yes	9.5
S3 (RM:anode-cathode)	0.74	Yes	No	6.2
S4 (AD:anode-cathode)	0.37	Yes	No	5.7

The low coulombic efficiencies shown in table 2 indicate that a substantial amount of the electrons reaching the cathode are being diverted to other purposes rather than acetate production. The presence of methane and/or hydrogen in the off-gas clearly indicates that some of these electrons end up in those gases. Unfortunately, gas flowrates could not be measured accurately enough to provide a confident quantification of the incidence of those “electron sinks”. In addition, as the microbial communities are on the start-up and proliferation stage, it seems reasonable to think that a significant amount of electrons is also being diverted to biomass production, all of which could explain the relatively low columbic efficiencies found in the present study.

3.2 Microbial community assessment

3.2.1 Diversity indices analysis

The number of quality reads per sample ranged from 5430 to 104,398 for eubacterial and from 1465 to 68,084 for archaeal communities. No microbial analyses were performed for the strategy S1 due to the absence of biofilm. The rest of the samples were rarefied to 500 sequences for a good comparison in diversity analysis. Despite

this reduction in sequence number, the richness and diversity of all samples were considered to be sufficiently covered. Regarding the eubacterial community, wide differences both in species richness indicators (observed OTUs and Chao1), and in diversity indicators (Shannon (H') and 1/Simpson) were found between the AD and RM inoculum (Table 3). Both indicators were much higher in the RM inoculum, as might be expected.

In S2, the diversity and richness indices decreased from **AD inoculum** (1/Simpson=33, Chao1=353) to **$S2_{13w}$_$AD_{cathode}$** (1/Simpson=17, Chao1=243), and in the same way these indicators decreased in the S3 from **RM inoculum** (1/Simpson=174, Chao1=426) to **$S3_{13w}$_$RM_{Cathode}$** (1/Simpson=3.0, Chao1=149). The same enrichment trend is observed in both cases, even sharper in the S3, due to the highly diversity of the **RM inoculum**.

However, in S4, the diversity remains constant from the **AD inoculum** (1/Simpson=33) to **$S4_{13w}$_$AD_{cathode}$** (1/Simpson=34), while the richness is almost three-fold higher in the **AD inoculum** (Chao1=353) compared to the **$S4_{13w}$_$AD_{cathode}$** biofilm (Chao1=141).

Table 3: Estimated richness (observed OTUs and Chao1) and diversity indices (Shannon (H') and 1/Simpson) for eubacterial operational taxonomic units (OTUs), calculated with MOTHUR at the 3% distance level.

Samples	Observed OTUs	Chao1		Shannon (H')		1/Simpson	
		mean	(c.i.)*	mean	(c.i.)*	mean	(c.i.)*
Strategy S2							
AD inoculum	211	353	296-447	4.3	4.2-4.4	33	29-38
$S2_{3w}$_$AD_{Cathode}$	112	163	136-223	3.4	3.3-3.5	13	11-15
$S2_{13w}$_$AD_{Cathode}$	117	243	179-373	3.5	3.4-3.6	17	15-19
Strategy S3							
RM inoculum	351	426	398-470	5.5	5.4-5.6	174	145-217
$S3_{3w}$_RM_{Anode}	184	315	259-414	4.3	4.2-4.3	38	34-43
$S3_{13w}$_$RM_{Cathode}$	63	149	98-272	1.8	1.7-1.9	3.0	2.8-3.3
Strategy S4							
AD inoculum	211	353	296-447	4.3	4.2-4.4	33	29-38
$S4_{3w}$_AD_{Anode}	225	351	301-436	4.6	4.5-4.6	46	41-54
$S4_{13w}$_$AD_{Cathode}$	103	141	118-200	3.9	3.9-4.0	34	31-38

*c.i. 95% confidence intervals

Results for archaeal analysis indices are presented in Table 4. Archaeal analysis was performed for the initial inocula and for the cathode biofilms at the end of the experiments. The numbers of sequences found were 52,490 and 68,084 for the **AD**

and **RM inocula** samples, respectively, and the quantity of archaeal decreases sharply to 1465 and 1473 sequences on the cathode biofilms for **$S3_{13W}$_$AD_{cathode}$** and **$S2_{13W}$_$RM_{cathode}$**, respectively. However, it should be highlighted that just eight sequences were found on the **$S4_{13W}$_$AD_{cathode}$**, indicating that the archaea population was inhibited under this condition.

Similar results to those found in eubacterial analysis were found for the archaeal population. In both strategies (S2 and S3), the diversity is between a two and three-fold higher in the initial inocula (**AD** and **RM inocula**) and decrease in the cathode biofilms (**$S2_{13W}$_$AD_{cathode}$** and **$S3_{13W}$_$RM_{cathode}$**). The richness indicator for S2 is lower in the **$S2_{13W}$_$AD_{cathode}$** (Chao1=35) than in the **AD inoculum** (Chao1=109), but in S3 this richness index is a four-fold increase over the **RM inoculum** (Chao1=322) than in the **$S3_{13W}$_$RM_{cathode}$** (Chao1=81).

Table 4: Estimated richness (observed OTUs and Chao1) and diversity indices (Shannon (H') and 1/Simpson) for archaeal operational taxonomic units (OTUs), calculated with MOTHUR at the 3% distance level.

Samples	Observed OTUs	Chao1		Shannon (H')		1/Simpson	
		mean	(c.i.)*	mean	(c.i.)*	mean	(c.i.)*
Strategy S2							
AD inoculum	61	109	80-183	2.2	2.1-2.3	4.2	4.0-4.6
$S2_{13W}$_$AD_{Cathode}$	16	35	21-92	1.1	1.0-1.2	2.4	2.3-2.5
Strategy S3							
RM inoculum	132	322	231-497	3.0	2.9-3.2	6.1	5.4-7.0
$S3_{13W}$_$RM_{Cathode}$	22	81	40-213	0.25	0.18-0.31	1.1	1.0-1.1
Strategy S4							
AD inoculum	61	109	80-183	2.2	2.2-2.3	4.2	4.0-4.6
$S4_{13W}$_$AD_{Cathode}$	No Archaeal found						

*c.i. 95% confidence intervals

3.2.2 Eubacterial community structure

Microbial community composition in the initial inocula used and growing on the surface of the carbon felt within the anode and cathode chamber were characterised by means of high throughput sequencing techniques.

RM inoculum presents a high diversity and it is composed of 12 different phyla, while in **AD inoculum**, which comes from a more specialised environment, *Firmicutes* (46.1%) is the predominant phylum (Fig. 2). The predominant phyla in all electrodes were *Proteobacteria*, *Bacteroidetes* and *Firmicutes.*

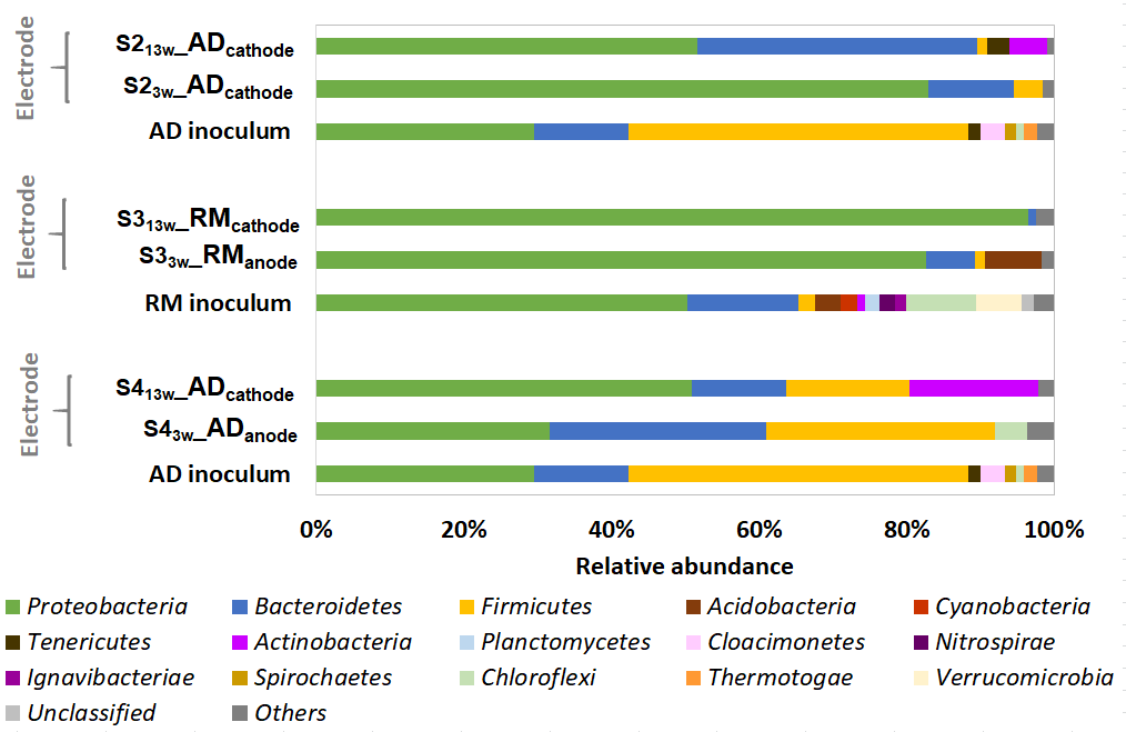


Fig. 2. Taxonomic classification of high throughput sequencing at phylum level.

To better understand the microbial community evolution for each strategy, Fig. 3 compares the families on the initial inocula and those that develop on the anode and cathode biofilms. As already mentioned, no microbial analyses were performed for strategy S1. Additionally, and to have an overview of the main species present on the biofilms, a heatmap is shown (Fig. 4).

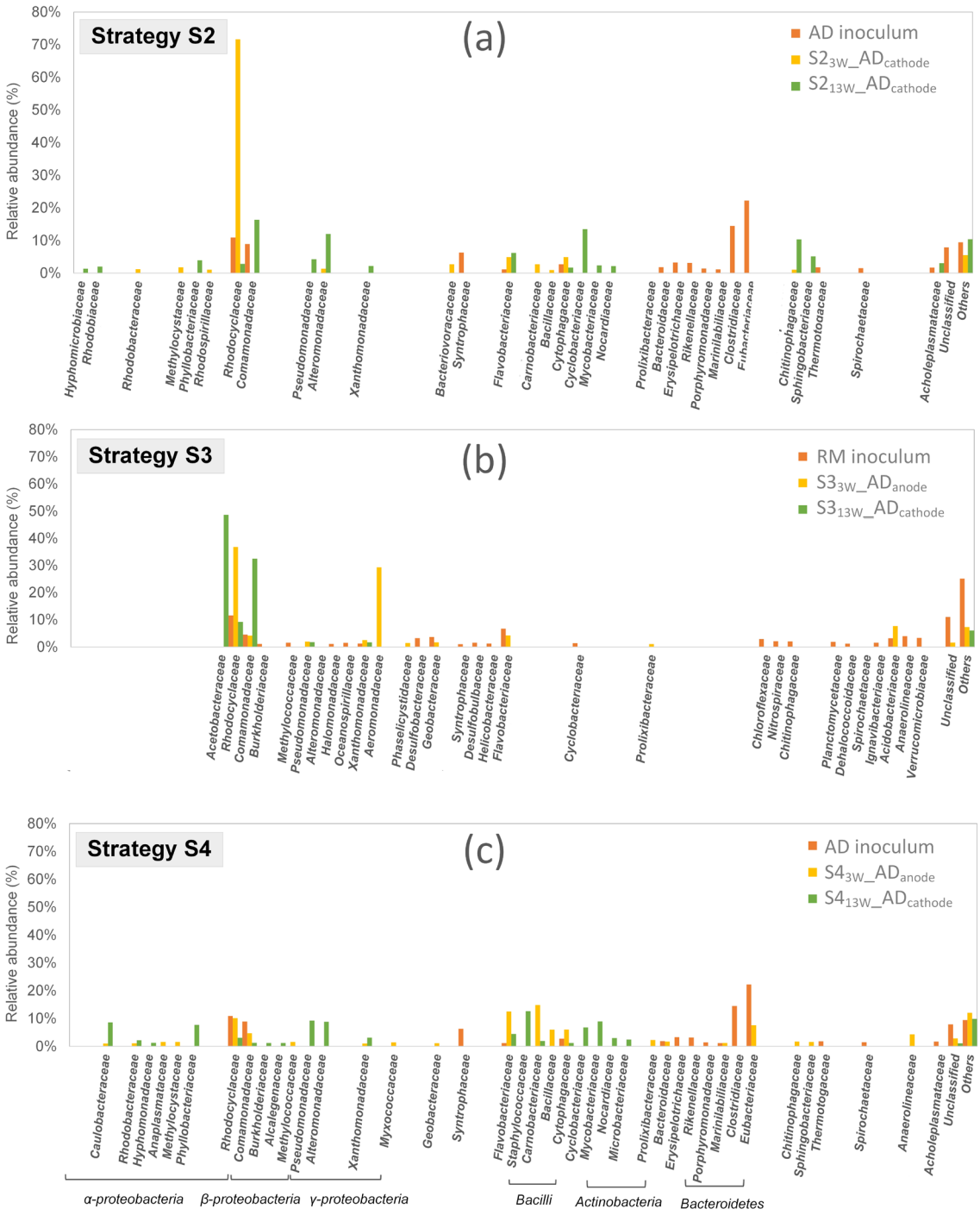


Fig. 3. Taxonomic classification of sequencing results of *16S rRNA gene* from eubacterial communities at a family level of a) samples from S2, b) samples from S3 and c) samples from S4. Groups accounting for less than 1% of the total number of sequences per sample were classified as 'others'.

The anodic microbial populations were dominated by syntrophic interactions of fermenters, homoacetogens and anode respiring bacteria (ARB) (Figs. 3 and 4). Within the ARB, the well-known *Geobacter* is found in all anodic biofilms, independently of the inocula used (Fig. 4); however, after the polarity was inverted in S3 and S4, *Geobacter*

was not identified. *Arcobacter,* a microaerobic electrogenic bacteria was found at the anode of S4 (first *ε-proteobacteria* demonstrated to act as exoelectrogen [30]). Apart of these species, another important microorganism, *Desulfobulbus,* known as cable bacteria [31], which are directly related with current generation in BES, was found at all anodes biofilms. Some microorganisms which have been previously enriched and described at cathodic biofilms, such as *Alishewanella* [32], and *Rhodococcus* [33] were also found in the cathode biofilms of S2 and S4 (Fig. 4).

In S2, a sharp enrichment in *Rhodocyclaceae* (72%) (a hydrogen producing bacteria [34]) occurred in just 3 weeks (**$S2_{3w}$_$AD_{cathode}$**). Our results showed that this family was mainly represented by two ribotypes, *Zooglea* (50%) and *Azoarcus* (19%) at week 3 of the experiment (Fig. 4). After 13 weeks of operation, the cathode microbial community becomes more diverse over time (**$S2_{13w}$_$AD_{cathode}$**) (Fig. 3a). The *Rhodocyclaceaea* family suffered a large decrease from 72% to 3%, while other families such as *Comamonadaceae*, *Alteromonadaceae*, *Pseudomonadaceae* and *Xanthomonadaceae* were enriched. The main genus of Comamonadaceae present was *Hydrogenophaga* (15%) (Fig. 4), which is an autotrophic hydrogen-oxidising bacteria [34]. Hydrogen-consuming microorganisms, such as *Hydrogenotropha* or *Thiobacillu*s, electrotrophic and also H_2 oxidising bacteria, were present.

For S3, where the electrodes were initially operated as anodes and inoculated with the highly diverse **RM inoculum**, the biofilms were swiftly enriched in nine anodophilic families (**$S3_{3w}$_RM_{anode}**) (Fig. 3b). The two predominant families were also *Rhodocyclaceae* (37%) and *Aeromonadaceae* (29%). When the WEs were turned into cathode, the biofilm (**$S3_{13w}$_$RM_{cathode}$**) became further specialised, with *Acetobacteraceae* (49.0%), *Comamonadaceae* (33.0%) and *Rhodocyclaceae* (9.2%) being the most abundant families. *Acetobacteraceae* belongs to the acetic acid bacteria (AAB), which can produce acetic acid using the Wood-Ljungdahl pathway oxidising H_2 and using CO_2 as electron acceptor [35]. This family is represented by a single genus, *Acetobacter* (49%) (Fig. 4). *Acetobacterium* (a homoacetogenic non-electroactive bacteria) was found in much smaller abundance (0.2%).

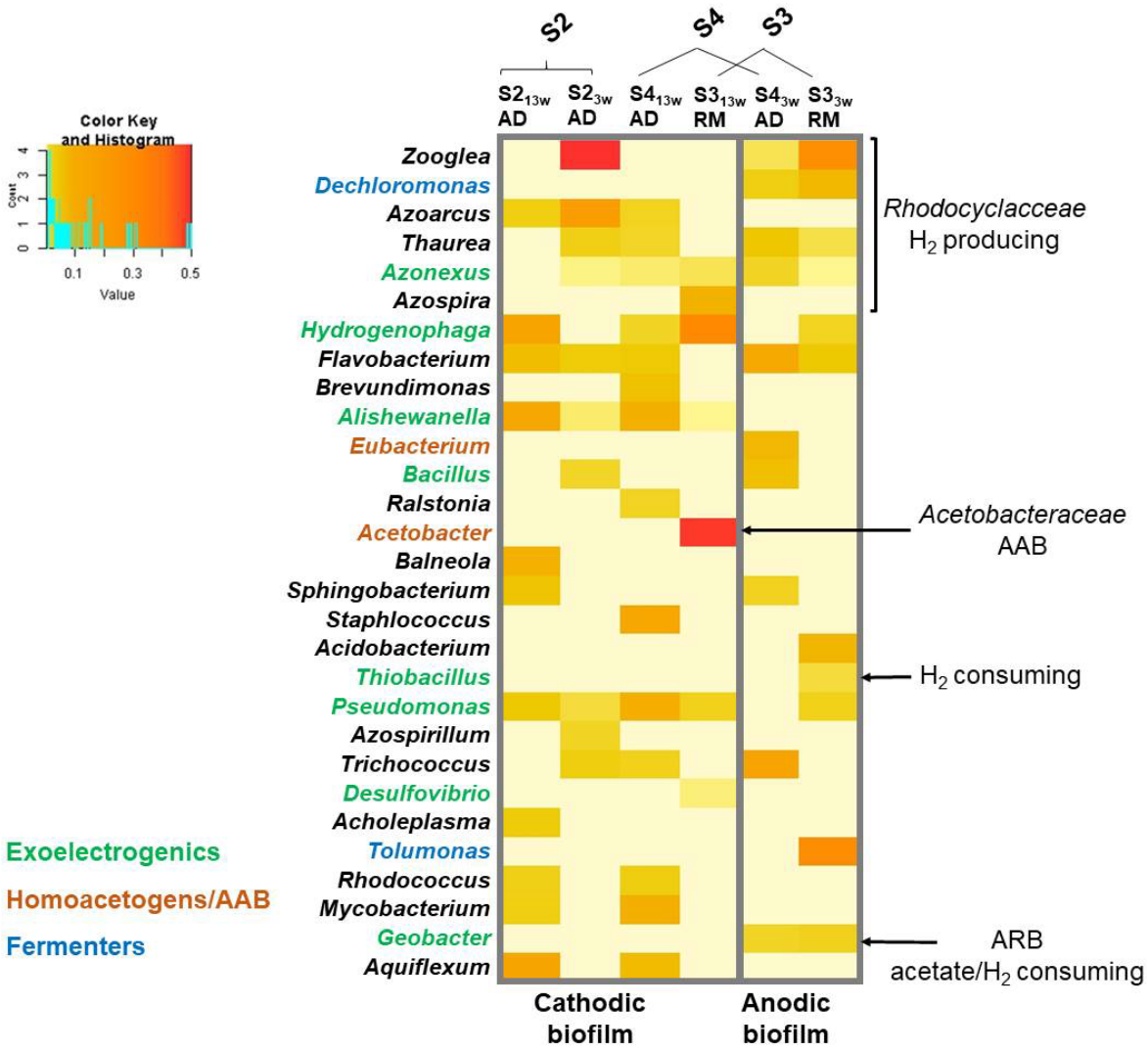


Fig. 4. Heatmap summarising the main genera present at the anode and cathode biofilms for the three strategies where a biofilm developed.

In strategy S4 (which followed the same start-up procedure as S3, but using **AD inoculum**), we found a drastically different scenario (Fig. 3c). A highly diverse biofilm was found in the anode (**$S4_{3w}$_AD_{anode}**), as well as when transformed into cathode (**$S4_{13w}$_$AD_{cathode}$**). Although **AD inoculum** is a highly specialised inoculum, the microbial community population that developed in the anodic biofilm sharply changed. Furthermore, when the anode was turned into a cathode, the biofilm population was very diverse as well, but completely different from the anode and also the inoculum.

3.2.3 Archaeal community structure

In general, archaeal communities display lower growth rates compared to eubacteria; thus only initial inocula samples and cathode biofilms samples taken after 13 weeks of

operation (once they were well stabilized) were analysed for archaeal community structure.

In both inocula (**AD** and **RM)**, the two predominant families were *Thermoplasmataceae* and *Methanosaetaceae*. Other families such as *Methanoregulaceae*, *Methanobacteriaceae*, *Crenarchaeota* and *Methanomassiliicoccaceae* were also found, although in a lower proportion (Fig. S2, supplementary information, section S3). Despite these similarities, the archaeal communities that developed on the cathode biofilms were drastically different (Fig. 5 and Fig. S2, supplementary information). Cathode biofilm in the S2 (**$S2_{13w}$_$AD_{cathode}$**), showed an important enrichment in the *Methanosaeta* (an acetoclastic methanogen belonging to the Methanosaetaceae family (49%)), and *Methanomassiliicoccus* (a methylotrophic H_2–dependent methanogen [36] that belongs to the Methanomassiliicoccacea family (44%)).

An important enrichment in the hydrogenotrophic methanogen *Methanobacterium,* which belongs to *Methanobacteriaceae* family, was observed on the cathode biofilm in S3 with respect to the **RM inoculum** (97% and 2.7%, respectively (Fig. 5)). As mentioned in the diversity indices section, no Archaeal population was found S4.

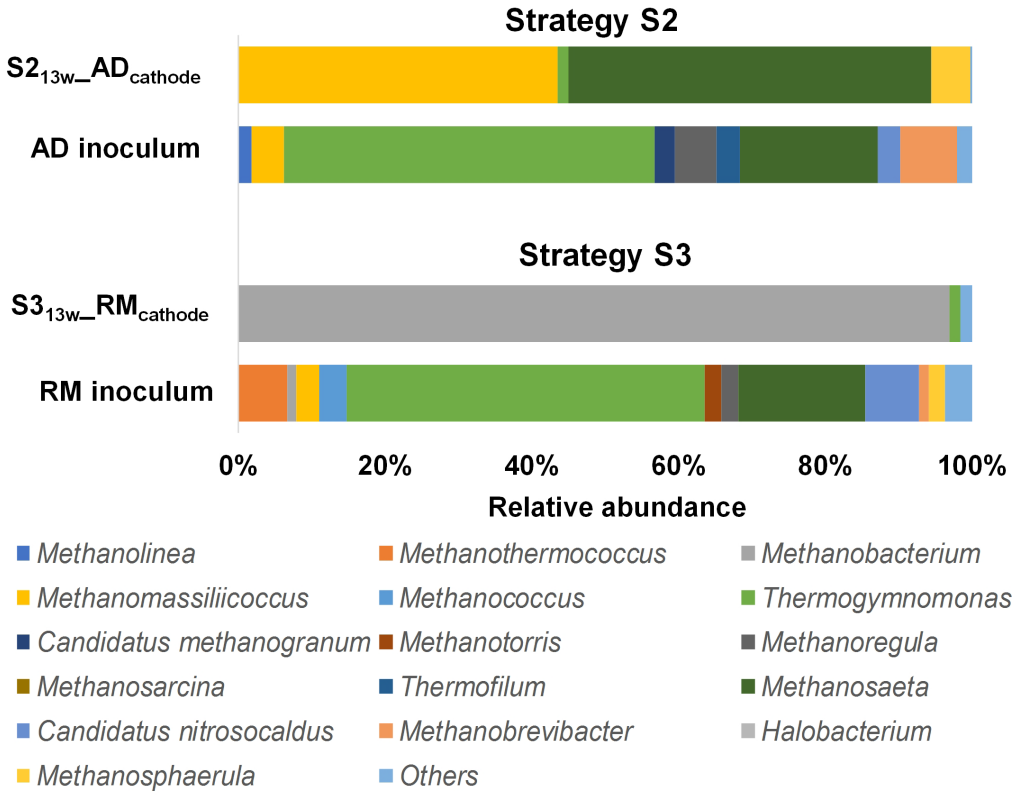


Fig. 5. Taxonomic assignment of archaeal microbial communities of AD and RM inocula, and cathode samples taken from S2 and S3 strategies at a genus level. Groups accounting for less than 1% of the total number of sequences per sample were classified as ‘others’.

4. Discussion

The results presented in this article seem to confirm a usual finding reported by other authors when producing biocathodes for microbial electrosynthesis: biocathodes struggled to form a viable biofilm by merely imposing reductive potentials. In our particular case this was so, regardless of the characteristics of the inoculum being used: strategy S1 (from a diverse inoculum) totally failed to produce any biofilm in any of the three replicates, and strategy S2 (from a lower diverse inoculum) required a 2-week lag-phase to develop an electricity-producing biofilm. A drastically different behaviour was observed when the bioelectrodes were started-up with oxidative potentials (strategies S3 and S4). For both strategies, the bioelectrodes started to produce an oxidative current in about 24 hours, and peak currents stabilised just after two cycles. When converted to biocathodes (by imposing reductive potentials) they required 3 to 4 days to produce an appreciable cathodic current, although we believe this 'lag-phase' can be partially explained by the microbial stress during microbiological sampling of the electrodes. Moreover, cathodic currents in strategies S3 and S4 tended to grow at a much higher rate than in S2. This promising behaviour observed in biocathodes started-up as bioanodes could be attributed to the rapid development of anode respiring bacteria (ARB) (*Geobacter* and Thiobacillus) and their subsequent syntrophic interactions with fermenters (*Dechloromonas* and *Tolumonas*) and homoacetogens (*Treponema*). These anodic microorganisms might be responsible for reaching a total degradation of the organic matter and obtaining good current production.

Interestingly, for all reactors and regardless of the inocula and the starting-up potential, the predominant phyla enriched in the electrode biofilms were *Proteobacteria*, *Bacteroidetes* and *Firmicutes*, which also confirms the observations made by other authors [34,37,38] (mainly in acetate fed MECs). *Proteobacteria* phylum contains well-known electrochemically active bacteria [39], and members of the classes α, β, γ and δ-*Proteobacteria* were identified in our electrodes. Furthermore, the proportion of this phylum tends to be raised in anode and cathode biofilms with respect to the initial inocula. To date, for *Bacteroidetes*, only two species have been claimed to be electroactive [40], and the vast majority of the species belonging to this phylum are not described as electrochemically active bacteria. However, its presence in BES is highly widespread, which suggests its importance for efficient biofilm function [37]. Despite these similarities at the phylum level, the results of this study indicated that greatly different eubacterial phylotypes were identified in each strategy carried out. It is

consistently highlighted that as the archaeal community is quite similar in both inocula, the dominant families on the cathode biofilms were drastically different. Apparently, the results point to a quicker enrichment in electrotrophic eubacterial communities using an anodic start-up. Below is a detailed summary of the main findings for each start-up strategy regarding biofilm development:

Strategy S1: combining cathodic start-up potentials with a diverse inoculum.

Although the inoculum contained various potentially electroactive bacteria, none of them succeeded in colonising the electrode. This is probably due to the fact that most of these bacteria oxidise organic chemical species and cannot modify their metabolic pathways to be viable at reductive potentials.

Strategy S2: combining cathodic start-up potentials with a low diverse inoculum.

This strategy showed a sharp enrichment during the first 3 weeks of operation, finding up to 70% of a H_2 producing family (*Rhodocyclaceae*). After 13 weeks, this family is still present in the biocathode, although its proportion is drastically reduced due to the proliferation of other cathodophilic families (*Hydrogenotropha and Thiobacillus*) responsible for H_2 oxidation and homacetogenesis. Regarding the archaea population, acetoclastic and hydrogenotrophic communities are present in a similar proportion. This microbial community evolution, together with steadily growing current records during the start-up period, suggests that electrotrophic H_2 producing bacteria firstly spread on the biocathode, generating a suitable environment for other cathodophilic bacteria responsible for acetic acid production. The spread of H_2-producing bacteria during the first phase of inoculation could also have paved the way for the proliferation of the H_2-dependent methanogen *Methanomassiliicoccus,* which could explain, at least in part, the presence of methane in the off-gas.

Strategy S3: combining anodic start-up potentials with a diverse inoculum.

Despite using the same inoculum as in S1, the outcome of this strategy is totally different, probably as a result the oxidative potential imposed in S3. During the first 3 weeks of operation, the electrode community becomes highly specialised in certain ARB bacteria (*Geobacter*, *Desulfovibrio* and *Thiobacillus*), achieving a complete substrate degradation. Interestingly, when the electrode potential was inversed (cathode operation mode), some of these electrogenic bacteria were maintained, and acetic acid producing bacteria such as *Acetobacter* and *Acetobacterium* enriched over 50% of the total population. Presumably, the non-strictly anaerobic environment in our

reactors can be responsible for the proliferation of these AAB against the typical homoacetogenic bacteria usually found in more strict anaerobic conditions [41,42]. It is important to note that this non-electroactive bacteria has been described as contributing to the microbial consortia via direct electron transfer (DET) [5], which could play an important role in the current production in this strategy.

Aside from ABB, acetate production could also be explained by the interaction between *Desulfovibrio and Acetobacterium. Desulfovibrio* belongs to *δ-proteobacteria* class, and is known to use sulfate as an electron acceptor, and is also able to grow converting formate into H_2 [12]. *Desulfovibrio* was only identified on this strategy, probably in synthropic conditions with *Acetobacterium,* and other microorganisms related to formate metabolism, since *Acetobacterium can* use formate to produce acetate [12]. *Desulfovibrio* can also act as acetogenic and produce acetate itself when the sulfate is in a low concentration and there is an H_2 / CO_2 atmosphere, as may be the case in our reactors. On the biocathode, biologically H_2 can also be generated by some bacteria identified in this strategy, which could favour the presence of hydrogenotrophic archaea as well as H_2 consuming acetogens. The detected H_2 might cause strong competition from hydrogenotrophic methanogens. For this reason, it is not surprising that an important enrichment in the hydrogenotrophic methanogen *Methanobacterium*, which belongs to the *Methanobacteriaceae* family, is observed on the cathode biofilm with respect to the RM inoculum.

Overall, these findings suggest that it is possible to develop a robust acetate-producing biocathode in a shorter period of time (compared to S2) from a working bioanode. This rapid response seems to be related to the anodic potentials during the start-up and the high bacterial enrichment.

Strategy S4: combining anodic start-up potentials with a low diverse inoculum.

This strategy uses an inoculum obtained from an anaerobic digester, which represents a low diversity inoculum as the microbial communities have been previously adapted to the specific conditions of anaerobic digestion. Interestingly, the microbial structure drastically changes and diversifies when introduced to the particular environment of a bioanode. Interestingly, this population is rather different from the anode in S3 but shows comparably good results in terms of current production and substrate degradation. However, when the potential of the electrode is inverted, few microbial families resist on the biocathode, promoting a complete rediversified community with

no single predominant family, which contrasts with the specific biocathode reached in S3. No archaea were detected, which agrees with the absence of detected methane. This non-specialised resulting community is capable of achieving a comparable but lower cathodic current than the previous S3; nevertheless, the lower acetic acid production found for this strategy suggests that a specialised biofilm is preferable.

5. Conclusions

This study elucidates the impact of the start-up strategies on the microbial communities that evolve on the biofilm of a biocathode. Using reductive start-up potentials and a highly diverse inoculum, this start-up failed to produce any biofilm. When a less species richness inoculum from an anaerobic environment was used with the same reductive initial potential, a specialised biofilm was formed and a highly productive biocathode was developed in terms of acetic acid and also current production. However, using oxidative start-up potential led to rapid electroactive biofilm development, although the final composition of the biofilm was highly dependent on the inoculum used. So, using the diverse RM inoculum, a final specialised biofilm grew on the electrode, also giving high acetate and current generation. However, when using the less species richness AD inoculum, it was found that a non-specialised biofilm was developed and lower acetic acid production was found.

Importantly, a higher specialisation of the biofilm leads to an improvement in acetate generation, probably due to lowered influence of undesirable secondary methabolic pathways. Moreover, it has been shown that the coupling of H_2 producing bacteria and acetic acid bacteria play an important role in acetate production.

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