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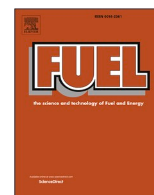

Full Length Article

Electromethanogenesis at medium-low temperatures: Impact on performance and sources of variability

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ABSTRACT

In this study we aimed to understand the impact of medium–low temperatures on the two main steps that usually comprise the electromethanogenesis (EM) process: electrotrophic hydrogenesis and hydrogenothrophic methanogenesis. Results revealed that pure CO_2 could effectively be converted into a high-purity biogas (~90:10 CH_4/CO_2) at 30 °C. However, when temperature was reduced to 15 °C, methane richness greatly decreased (~40:60 CH_4/CO_2). This deterioration in performance was mostly attributed to a decline in methanogenic activity (represented mainly by *Methanobacterium* and *Methanobrevibacter)*. In contrast, the hydrogenic activity (mostly *Desulfomicrobium)* did not suffer any significant decay. Results also seemed to indicate that methanogenesis, rather than hydrogenesis, is the main source of variability in EM. Increasing the temperature again to 30 °C restored previous performance, which highlights the resilience of EM to wide temperature fluctuations (from 30 to 15 and back 30 °C).

1. Introduction

The term bioelectrochemical systems (BESs) encompasses a family of electrochemical devices that use microorganisms as pseudo-catalysts to promote anodic and/or cathodic reactions [1]. The definitive take-off in BES research came in 1999 with the discovery that certain types of microorganisms (usually known as electrogens) could exchange electrons with the anode (bioanode) without the need of a redox mediator [1–3]. In the following years, research interest in BES expanded further from bioanodes towards biocathodes, when it was found that a group of microorganisms, usually termed as electrotrophs [4], can catalyse a wide range of (bio)cathodic reactions, such as hydrogen formation [5], oxygen reduction [6], nitrate and nitrite reduction [7], or the production of organic compounds [8]. Among the latter, the use of BESs for methane production from CO_2 has aroused noticeable interest because of its environmental and economic potential [9]. This process, usually termed electromethanogenesis (EM), can proceed at room temperatures and pressures and involve bacteria as a catalysts, all of which suggest that EM can become a more cost-effective and environmentally friendly method of methane production compared to conventional technologies [10]. Despite that, technical and economic limitations still remain, and the scaling up of this technology represents a major challenge [11].

The production of methane through EM depends on factors such as reactor configuration (e.g., membrane vs membraneless configurations) [12], and on various operating parameters, such as cathode potential, pH, temperature, buffering capacity or the composition and concentration of the catholyte [13]. Among them, temperature plays a key role as it has a direct impact not only on the process in itself (i.e., the metabolic routes and the microbial dynamics) [14] but also on its economics (i.e., the higher the temperature, the higher the energy requirements for heating). Regarding the metabolic routes of EM, previous studies have found strong evidence that methane formation occurs through a two-step process, in which electrotrophic hydrogen production is coupled to hydrogenotrophic methane formation [15,16]. It is also widely known (from studies on microbial ecology, anaerobic digestion, or biological hydrogen methanation), that temperature has a significant impact on hydrogenotrophic methanogenesis, affecting both the methane productivity and the microbial community structure [17–19].

Despite that, little attention has been paid so far to the effect of temperature on EM. To the best of our knowledge, only one study, published in 2018, deals with this issue [13]. In a previous work by Wang *et al.* [20] it was shown that when a biocathode is brought from 25 to 9 °C in a membraneless BES, methane production immediately stops, while the hydrogen content in the off-gas increases significantly.

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However, this cannot be properly considered an EM study, since the biocathode was aimed at generating hydrogen, and methane was a secondary undesired product. In comparison, Yang *et al.* [13] explored the influence of temperature on methane production using an EM system in a strict sense. The authors operated a mixed-culture biocathode in a wide range of temperatures (between 15 and 70 °C), showing that the performance of the EM system can be optimised at around 50 °C. However, this is a relatively high temperature that may impose large heating requirements on a potential full-scale system, thus compromising its economic feasibility.

In our paper, we explore the capability of EM to convert pure CO_2 into methane in a milder temperature range (between 15 and 30 °C), paying special attention to the conversion rates and the quality of the biogas produced. We also try to gain insight into the impact of temperature on the two main stages of EM (i.e.: electrotrophic hydrogenesis and hydrogenotrophic methanogenesis), making a preliminary effort to understand the sources of variability in EM.

2. Materials and methods

2.1. Reactor design

Two standard H-type reactors, with an internal volume of 500 mL per chamber, were used as reactor vessels. They were named as U1 and U2 and were intended to be replicates. Each of the working electrodes (cathodes) consisted of two 2 × 8-cm carbon felt electrodes (SGL Group, Germany) joined by a titanium wire and suspended inside the cathodic chamber (total projected area of 0.005 m^2). The counter electrodes (anodes) were of 2 × 2-cm platinum mesh (Goodfellow, UK) suspended inside the anodic chamber with a titanium wire. Prior to inoculation, the cathodes were pre-treated by subsequent immersion in 1 M nitric acid, 1 M acetone and 1 M ethanol for 24 h each to avoid hydrophobicity and remove impurities [21]. The anodic and cathodic compartments were separated by means of a cation exchange membrane (CMI7000, Membranes International, USA).

Both units were operated in a three-electrode configuration at an applied potential of -1 V vs Ag/AgCl (–0.8 V vs. SHE). The stability of the reference electrode (Sigma-Aldrich, USA) was checked prior to every batch cycle. All the electrochemical tests were performed with a Biologic VSP potentiostat assisted by EC-Lab software (v11.30).

The catholyte was stirred continuously using a magnetic stirrer at 200 rpm to prevent mass transfer limitations [22].

Appropriate connections and sealing were designed for sampling and substrate supply. CO_2 was fed to the cathode with the help of a 1-L bag (Ritter, Germany). This same bag was used to collect the biogas produced.

2.2. Electrolytes

The anolyte consisted of 0.1 M potassium phosphate buffer in deionised water. The catholyte consisted of 20 mM potassium phosphate buffer, macronutrients (280 mg·L^{-1} NH_4Cl, 5.7 mg·L^{-1} $CaCl_2$, 10 mg·L^{-1} $MgSO_4{\cdot}7H_2O$, and 90 mg·L^{-1} $MgCl_2{\cdot}6H_2O$), 1 mL·L^{-1} of a micronutrient solution, and 1 mL·L^{-1} of a vitamin solution as described in [23]. $NaHCO_3$ (5 g·L^{-1}) was added as a carbon source since at pH 7. CO_2 was predominantly present as bicarbonate [23].

2.3. Inoculum and start-up

Anaerobic sludge obtained from an anaerobic digester at a local wastewater treatment plant was used as primary inoculum (1:5 proportion), as described before [24]. Although single species can provide better controlled environments and higher specific production, mixed cultures offer higher flexibility and resilience [25].

The whole start-up lasted approximately 55 days. During this period the reactors were operated in batch mode (see next paragraph for the operation procedures). A total of eight batch cycles were completed before the current stabilised (stabilisation was assumed after two consecutive cycles in which mean current density fluctuated by $< 10\%$).

2.4. Operation

From day 55 on (i.e., after the start-up period), a series of three tests intended to evaluate CO_2 conversion at 30 °C were carried out (Fig. 1). At the beginning of each test, 400 mL of CO_2 was added to each unit with the aid of a gas bag. Reactors were operated in batch mode, and the electrolytes were replaced every 4–5 days to avoid any limitation deriving from a lack of nutrients or from pH shifts. The duration of each batch cycle was determined by the measured current density: once this parameter fell below 30%, the cycle was stopped and a new cycle began.

Following these tests, temperature tests were conducted in a similar way at temperatures of 25, 20 and 15 °C. Temperature was kept constant (±1 °C) by thermoregulated bath (Digiterm 100 Selecta) in an unheated room.

Once this set of experiments was finished, biofilm samples from the cathodes were taken for microbiological analysis (Section 2.6) and a new cycle of experiments at 30 °C conducted to assess the resilience of the EM to medium–low temperatures.

2.5. Analytical techniques

Liquid samples were analysed for total organic carbon (TOC), total inorganic carbon (IC), total nitrogen (TN; Multi N/C 3100, Analytikjena) and volatile fatty acids (VFAs) from C_2 to C_6 [Bruker 450-GC with a flame ionisation detector (FID)]. Dissolved oxygen (Hach, HQ40d two-channel digital multimeter), redox (pH Meter, pH 91; Wissenschaftlich Technische Werkstätten, WTW), pH (pH Meter BASIC 20+, Crison) and ammonium (781 pH/Ion Meter, Metrohm) were measured following standard methodologies [21].

At the end of each batch cycle, the gas bag was disconnected from the reactor and the amount of gas in the bag (V_g) was measured with the aid of a gastight syringe (50 mL, Hamilton SampleLock syringe). Gas composition, i.e., hydrogen (H_2), carbon dioxide (CO_2), oxygen (O_2), nitrogen (N_2) and methane (CH_4), were determined by a gas chromatography (Varian CP3800 GC) equipped with a thermal conductivity detector (TCD) [21]. The volume of hydrogen and methane produced in each cycle was calculated from V_g and the gas mole fraction in the gas bag, and was corrected to the standard temperature and pressure (STP) conditions.

The electrochemical performance of the biocathodes was characterised by means of cyclic voltammetry (CV) tests using a Biologic VSP potentiostat. CV tests were performed in turnover and non-turnover conditions (i.e., in the presence and absence of CO_2 respectively) between − 1.0 and 0.1 V vs. Ag/AgCl and at a scan rate of 1 mVs^{-1} at a temperature of 30 °C.

The columbic efficiency (CE), which can be defined as the efficiency of electron capture from the electric current by methane, was calculated according to Equation 8 from ref. [26].

2.6. Molecular biology techniques

At the end of the temperature tests, the cathode was cut into samples of about 300 mg of electrode. These samples were used to characterise the microorganisms that had developed at the methane-producing biocathode.

Microbial communities were analysed in terms of Total Bacteria and Archaea. Genomic DNA was extracted with a DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. All PCR reactions were carried out in a Mastercycler (Eppendorf, Hamburg, Germany), and PCR samples were checked for size of product on a 1% agarose gel and quantified by NanoDrop 1000 (Thermo Scientific). The entire DNA extract was used for high-throughput sequencing of 16S-rRNA gene-

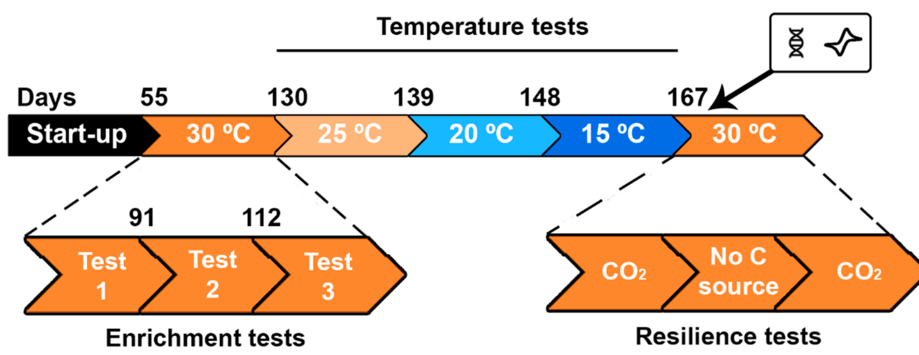

Fig. 1. Experimental timeline.

based massive libraries with 16S-rRNA gene-based primers: for eubacteria 27Fmod-519R and for Archaea 349F-806R at MR DNA (www.mrdnalab.com, Shallowater, TX, USA), utilising MiSeq equipment (Illumina, San Diego, CT, USA). DNA reads were compiled in FASTq files for further bioinformatics processing. The trimming of the 16S-rRNA bar-coded sequences into libraries was carried out using QIIME software, version 1.8.018 [27]. Quality filtering of the reads was performed at Q25 quality prior to grouping into operational taxonomic units (OTUs) at a 97% sequence homology cut-off. Subsequent steps were performed using QIIME, a denoising procedure using a denoiser algorithm [28]. Final OTUs were classified taxonomically using BLASTn against a database derived from the Ribosomal Database Project II (RDPII, http://rdp.cme.msu.edu) and the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov).

The quantitative analysis of all samples was carried out by means of quantitative-PCR (qPCR) using PowerUp SYBR Green Master Mix (Applied Biosystems) in a StepOnePlus Real-Time PCR System (Applied Biosystems) as described previously [24]. The qPCR amplification was performed for the 16S-rRNA gene in order to quantify the entire eubacterial community and for the mcrA gene to quantify the total methanogen community. The primer sets 314F qPCR (5′-CCTACGG-GAGGCAGCAG-3) and 518R qPCR (5′-ATTACCGCGGCTGCTGG-3′) at an annealing temperature of 60 °C for 30 s was used for Bacteria and Arc 349F (5′-GYGCASCAGKCGMGAAW-3′) and Arc 806R (5′-GGAC-TACVSGGGTATCTAAT-3′) for Archaea quantification.

3. Results and discussion

Following inoculation, current density increased steadily in both replicates (denominated as U1 and U2), reaching a maximum averaged current density of ~ 2 A/m^2 in Cycle 6 (Fig. 2). In the following two cycles (7 and 8), the current tended to stabilise around this value, so we assumed that the biofilms were mature enough to initiate the experimental period. It is important to note that during these eight cycles that covered the start-up period (spanning a total of 55 days), current density was reasonably similar in both reactors, thus showing good replicability. The large fall observed in reactor U2 during Cycle 4 was due to a power shortage; nevertheless, current resumed again in the following cycle.

3.1. CO_2 conversion tests

Following the start-up period, three consecutive CO_2 conversion tests (designated as Tests 1, 2 and 3 respectively) were conducted to assess the ability of both biocathodes to convert CO_2 into biomethane (Figs. 2 and 3). Temperature was maintained at 30 °C, and the gas bag of each reactor was filled with 400 mL of CO_2 at the beginning of every test. To

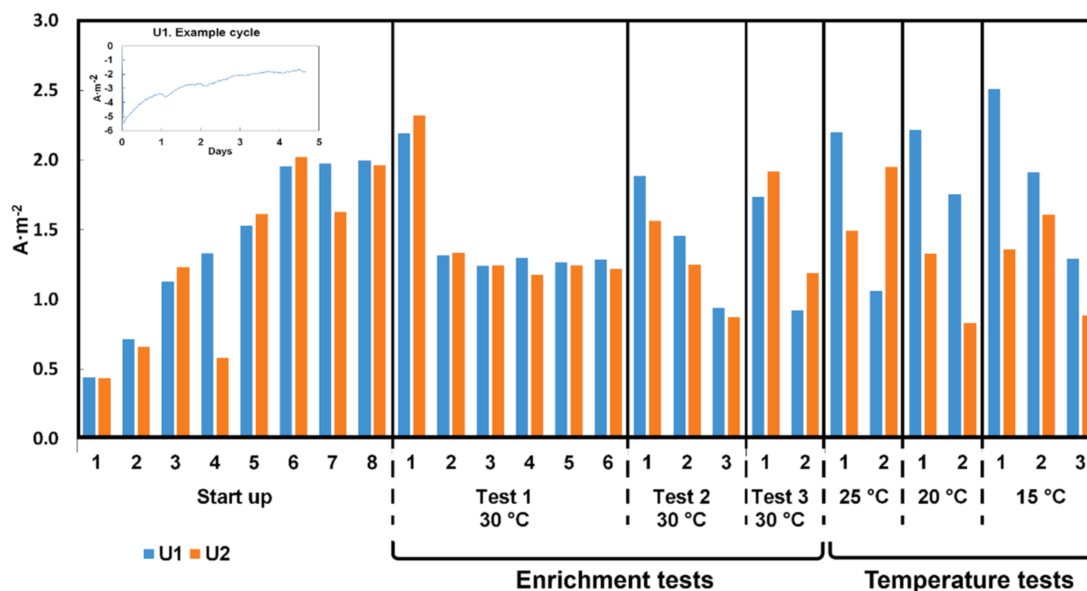

Fig. 2. Current density produced by reactors U1 (orange) and U2 (blue) during each batch cycle. The cycle number for the start-up, enrichment tests and temperature tests is also indicated. The inset of the figure shows an exemplary current density profile for a typical batch cycle. Columns represent the averaged current density for each individual cycle (computed as the mean value of the current density profile over that particular cycle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

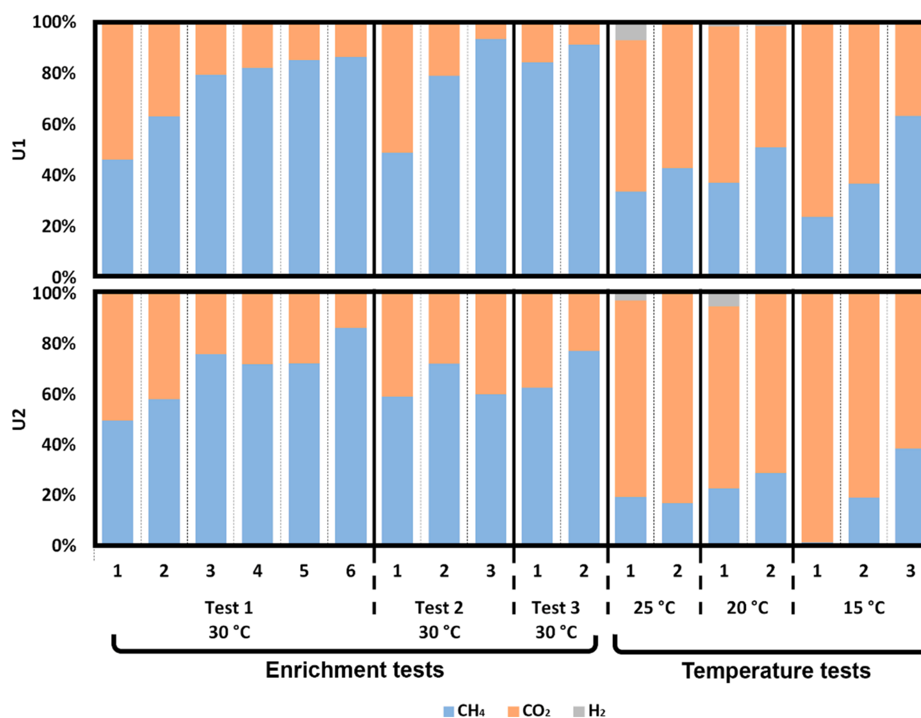

Fig. 3. Biogas content during CO_2 conversion tests (Tests 1, 2 and 3) and temperature tests (temperatures of 25, 20 and 15 °C).

make sure that nutrients and/or pH shifts were not limiting the conversion process, the catholyte and the anolyte were renewed with every cycle. The duration of each cycle was determined by current density: once this parameter fell below 30% of its maximum value, the cycle was stopped (see the inset in Fig. 2 for the current profile of a typical cycle). This resulted in a cycle duration of between 4 and 5 days.

Results revealed that after three consecutive cycles the methane content in the biogas tended to stabilise at around 80% in U1 and 60% in U2 (Fig. 3). The highest rate of conversion always occurred during the first cycle of every test, where the amount of methane increased up to 40%. In subsequent cycles this level fell to below 30%, probably as a result of the declining proportion of CO_2 in the gas bag. This is consistent with the fact that the highest coulombic efficiency (Table 1) was almost always obtained during the first cycle of each experimental phase. The only exception occurred in U2 during Phase 1. Analogous results have been reported [29] using an H-type cell (similar to those used in this study). In the referred work, methane production was measured over a period of four cycles (200–300 h each cycle), increasing dramatically between the first and second cycles (from 0.54 to 3.31 mmol CH_4). From these results, it seems that the optimal residence time to maximise methane production rate by EM might be 2–3 cycles (12–15 days). This may seem a rather large time period, especially if we compare it with the typical residence times of other CO_2 methanation processes such as the Sabatier process. However, we must keep in mind that EM, in contrast to the Sabatier process, proceeds under near-ambient conditions and that the current EM reactor vessels were conceived for laboratory research. Future improvements in reactor design (by, for instance, optimising the ratio of cathode surface area to catholyte volume) will undoubtedly result in substantial reductions in residence time. Thus, we can understand these figures as a benchmark against which to compare future developments.

Marked differences were also observed between the current density produced during the first and subsequent cycles (Fig. 2). While current density in the first cycle was around 2 $A \cdot m^{-2}$ in both reactors, it fell to below 1.5 $A \cdot m^{-2}$ in the subsequent cycles. This pattern can also be linked to the evolution of CO_2 in the gas bag: as the enriching process proceeds and the amount of available CO_2 diminishes, the pH tends to increase (results not shown), thus reducing the concentration of available H^+ in the catholyte.

Another important issue has to do with the divergent behaviour observed between U1 and U2 (Fig. 3), which tended to amplify with time: while methane proportion grew comparatively much faster in U1 (especially in Tests 2 and 3) reaching concentrations of up to 90%, it kept well below 80% in U2. Interestingly, and despite that, current density remained quite similar in both reactors during the same period (Fig. 2). This suggests a decoupling between electron consumption (current) and methane formation, indicating that EM might be proceeding in a two-step process in which hydrogen acts as an intermediary between current consumption (electrothrophic hydrogenesis) and methane formation (hydrogenothrophic methanogenesis), as already suggested in a previous study [16]. Acetate can be ruled out as an intermediary for the reasons that will be discussed below (Section 3.3). In addition, these results show that some processes explain better than others the natural variability of a BES. In our case it seems that the

Table 1
Coulombic efficiencies.

	Cycle	U1	U2
Phase 1 30 °C	1	51%	35%
	2	23%	50%
	3	8%	6%
	4	8%	0%
	5	22%	0%
	6	27%	35%
Phase 2 30 °C	1	65%	20%
	2	57%	17%
	3	24%	13%
Phase 3 30 °C	1	53%	19%
	2	0%	4%
25 °C	1	28%	5%
	2	13%	0%
20 °C	1	43%	14%
	2	13%	10%
15 °C	1	30%	0%
	2	4%	21%
	3	29%	8%

electrotrophic processes are much more reproducible than the methanogenic ones. This is something worth investigating in future studies, not only because it will enhance our understanding of microbial dynamics of BESs, but also because it can potentially advance this technology towards commercial application. We must keep in mind that variability introduces a risk in the commercialisation of any technology that may hamper business development [30].

3.2. Temperature tests

When temperature was set at 25 °C, the proportion of methane in the biogas declined sharply in both reactors (Fig. 3). Further reducing the temperature to 20 °C did not result in any significant difference compared to 25 °C. However, at 15 °C methane concentration once again experienced a decline during the first cycle (more visible in U2), although it quickly resumed (even exceeding) the values observed at 20 and 25 °C. In contrast to methane—and contrary to expectations—current density not only did not decline at low temperatures but even increased in some cycles. These observations might be evidencing important features of the dynamics of the microbial populations involved in EM that would deserve some attention in future research. First of all, and most importantly, these results seem to indicate that low temperatures have a quite different impact on the microbial communities that thrive on the cathodic environment of EM, with the microorganisms responsible for methane production being more negatively affected than those involved in hydrogen production.

These results also seem to indicate that after the initial shock (when temperature was brought to 25 and 15 °C), methanogenic communities are able to thrive in the new conditions. The fact that electrotrophic microorganisms endured the temperature drop more successfully (as evidenced by the current density profiles) than methanogens might have a close connection to the re-adaptation of methanogens. In this regard, it can be hypothesised that even at low temperatures electrotrophs can provide a continuous stream of hydrogen that might be vital for the recovery of methanogenic activity. The 'good health' of electrotrophs and the poor initial performance of methanogens is further evidenced by the presence hydrogen in the biogas immediately after the temperature was reduced (Fig. 3).

Finally, it is important to note that the differences in performance observed at 30 °C during the biogas enriching tests (see Section 3.1) were accentuated as the temperature was decreased. Even the current density, which had showed a remarkable replicability to this point, started to show large discrepancies between both reactors.

3.3. Understanding the different behaviours of U1 and U2

This large variability, typical of BESs in general and of EM in particular, impacts negatively the commercial prospects of these technologies, as mentioned before. To further investigate the observed differences in the behaviour of our systems, CV tests and biological analyses were performed at the end of the temperature tests.

CV analyses were performed in the presence and absence of the carbon source (CO_2) (Fig. 4). The greatest difference between U1 and U2 was found in the catalytic wave associated with hydrogen evolution (starting at about −0.8 V [31]), which was distinctly greater for U1, which could explain the higher current density observed in this reactor. When no carbon source was present (and thus the methanogenic hydrogen usage was inhibited) the differences between U1 and U2 were more apparent, with U1 showing a larger oxidation peak at −0.1 V that might be related to the oxidation of redox active compounds excreted by microorganisms [32], or even to hydrogen oxidation [33]. The presence of sulphate-reducing bacteria in our biocathodes (see below), capable of catalysing hydrogen evolution and oxidation reactions on solid electrodes, would be in favour of the latter [33].

Samples of the cathode biofilm from both units were taken for qPCR analysis. Results showed that the cathodic environments promoted a strong selection in favour of Archaea which, in contrast to Bacteria, experienced a remarkable increase in their gene copying (Fig. 5). However, the Archaea community presented a lower diversity than that of Bacteria, all of which is consistent with previous observations in EM systems [34].

Regarding Archaea, the biofilm was clearly dominated by the genus *Methanobacterium*, with a relative abundance of 84% and 69% in U1 and U2, respectively (Fig. 6). Most members of this genus live on the reduction of CO_2 with H_2 to produce methane, which is coherent with the hypothesis that H_2 acts as an intermediary in the electron transfer between the electrodes and CO_2. Another hydrogenothrophic genus found in the biofilms—although in far lower abundance—was *Methanobrevibacter* (2% and 4% for U1 and U2, respectively). These results

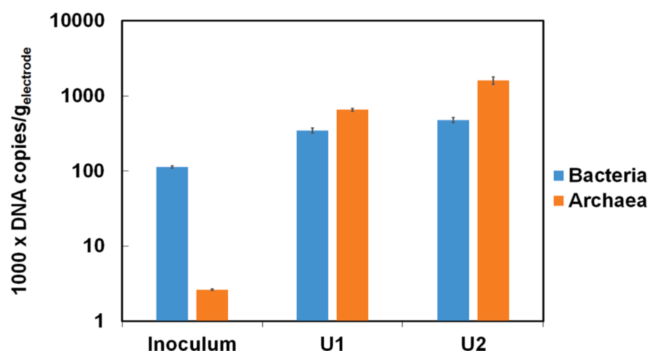

Fig. 5. Number of DNA copies per g of electrode for Bacteria and Archaea in qPCR analysis.

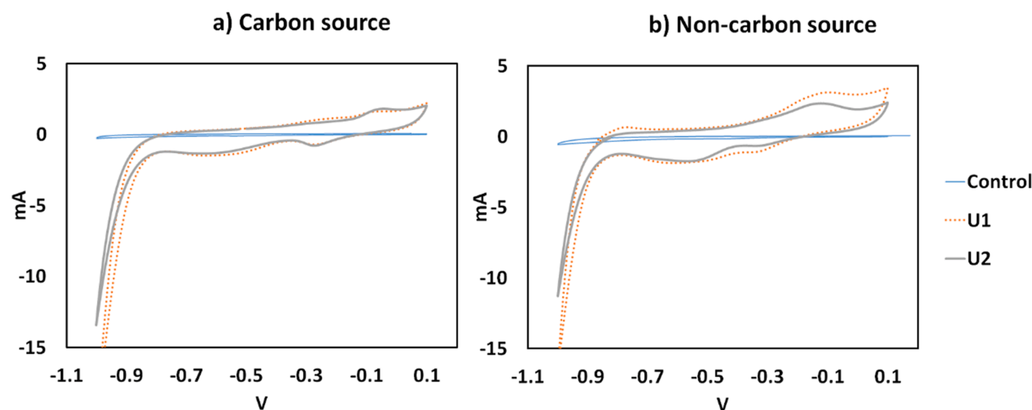

Fig. 4. Cyclic voltammetry with carbon source (a) and with no carbon source (b). An unmodified (with no biocatalysts) carbon felt electrode was used as control.

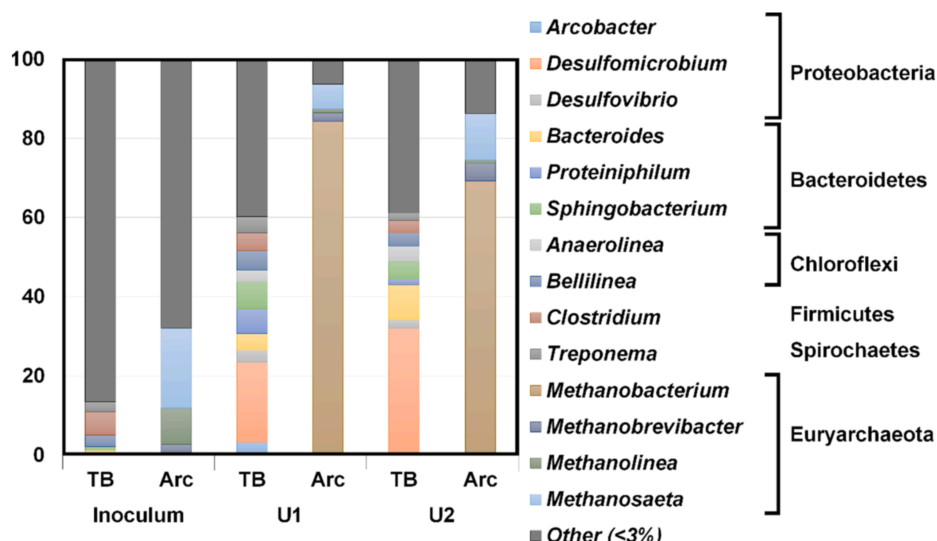

Fig. 6. Relative abundance for Total Bacteria (TB) and Archaea (Arc) of inoculum and biocathodes (U1 and U2) after the experimental period in terms of genus (first column) and phylum (second column). Only genera with at least a 3% abundance have been considered.

coincide with previous studies where the microbial communities of EM biocathodes were usually dominated by species of hydrogenotrophic methanogens regardless of the sources of inoculum [25,35], thus confirming the role of hydrogen as the main intermediary in EM. Other potential intermediaries such as acetate can be discarded, as TOC analyses of the medium resulted in values below 15 mg·L^{-1} during the tests at 30 °C. Even when the temperature was reduced to 25, 20 and 15 °C, and contrary to expectations, no appreciable amounts of any fatty acids were detected. It is known that acetoclastic methanogens are often more temperature-sensitive than hydrogenotrophic methanogens [12] and therefore, as a result of their lower metabolic activity, it would have been expected to find acetic acid in the medium. This is consistent with the decline in the relative abundance of genera such as *Clostridium* and *Proteiniphilum,* which are often related to acetic [36] and propionic acid [37] production. Probably as a result of this, acetolastic methanogens did not find a favourable environment for their proliferation, thus explaining the significant decline in their relative abundance, falling from 20% in the inoculum to 6% and 12% in the cathode biofilms of U1 and U2, respectively. This observation is somehow coherent with the results reported by Liu *et al.* [38], where the abundances of hydrogenotrophic methanogens in a biocathode operated at 20–25 °C increased significantly from those in the inoculum (anaerobic digestion sludge), while acetotrophic methanogens showed only a smooth increase.

Another key role that bacteria play in EM is the promotion of strictly anoxic conditions at the biocathodes (either by catalysing hydrogen formation or by oxygen scavenging) which are essential for the proliferation of methanogens [12]. In our cathodes we found a strong selection for hydrogen-producing bacteria, especially of the genus *Desulfomicrobium,* which experienced a remarkable increase in its relative abundance from<0.1% in the inoculum to 20.2% and 31.3% in reactors U1 and U2, respectively. Other genera, such as *Desulfovibrio*, which has been reported to be able to catalyse hydrogen production at cathode potentials more negative than –0.44 V vs SHE [23], and *Sphingobacterium* and *Anaerolinea*, capable of using the electrons arriving at the cathode to catalyse reductive process such as hydrogen evolution [6,7,39–41] were also found in significant relative abundances.

Overall, these results seem to confirm hydrogenotrophic Archaea to be primarily responsible for methane production. From a very diverse initial community in the inoculum, *Methanobacterium* and *Methanosaeta* stood out significantly, being supported by a strong presence of hydrogen-producing bacteria. In addition, as the growth medium was completely renewed at the beginning of every batch cycle, both methanogenic and hydrogenic microorganisms are more likely to grow on the cathode surface rather than forming planktonic cultures. Finally, although the divergent trend in biogas composition between U1 and U2 can be partially explained by the already discussed differences in the relative abundance of methanogenic Archaea, this is by no means the only plausible cause. It is probable that other genera that remain unnoticed because of their much lower relative abundance (the level of unidentified Archaea genera in our reactors was around 10%) might be playing key metabolic roles. Another possible explanation might be related to stochastic and uncontrollable phenomena that take place during the inoculation process and result in different structures of the biofilm (stratification) or diverging colonisation patterns (surface vs inner colonisation of the porous electrode).

3.4. Resilience tests

Following the electrode sampling for microbiological analysis, a final experiment at 30 °C was conducted to assess the resilience of EM to the temperature shocks undergone in the previous experiments. Surprisingly, and despite the traumatic event associated with the sampling process (i.e., scratching the cathodic biofilm) both reactors displayed a similar performance to that observed before the temperature tests (compare Cycles 1 and 2 in Fig. 7 with the results at 30 °C in Figs. 2 and 3). It is important to note that no re-acclimation period was allowed, and so the previous performance was resumed almost immediately from the first cycle.

Removing CO_2 from the feeding did not have a significant impact on the current density (Cycles 3 and 4 in Fig. 7) and, as expected, the off-gas consisted mainly of hydrogen. Nevertheless, a small amount of CH_4 (<25%) and traces of CO_2 were also detected, although they tended to disappear in the following cycle. This CH_4 can probably be attributed to the reduction of the CO_2 stored as small deposits of carbonates precipitated on the cathode surface in previous cycles (probably as a result of the locally high pH).

When CO_2 was fed again (Cycles 5 and 6), methane reappeared in the off-gas at similar concentrations to those observed in Cycles 1 and 2, indicating that the methanogenic activity was not significantly influenced by the CO_2 cut-off. Overall, these tests confirm the hypothesis of the two-step process and highlight the resilience of both the electrotrophic and the methanogenic communities to medium–low

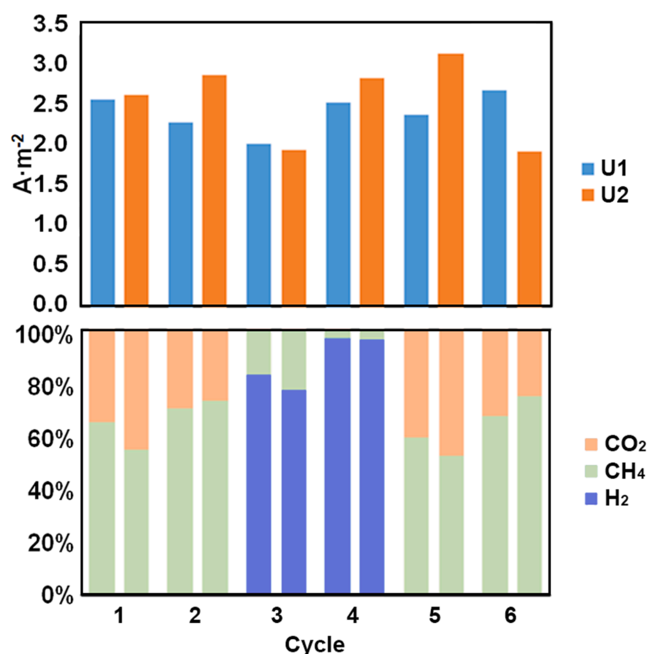

Fig. 7. Current mean (above) and gas proportion (below) during CO_2 cut-off test.

temperature conditions.

Finally, it should be noted that these tests were carried out using pure CO_2 as a substrate. It is still uncertain how the electromethanogenic biofilms would respond to the presence of toxic gases in the feed. The 2D biofilms (such as those used in this study) are environmentally sensitive and can prove to be unsuitable for applications such as biogas upgrading, where the presence of hydrogen sulphide in the biogas can be toxic for the cathodophyllic microorganisms [42]. In this regard, the use of 3D cathodic biocatalysts such as granular anaerobic sludge (more tolerant to environmental stresses [42]), can be an interesting alternative to conventional 2D cathodic biocatalysts [42].

4. Conclusions

In this paper we explored the capability of EM to convert CO_2 into methane in a wide range of temperatures (from 30 to 15 °C). Results indicate that although biocathodes can absorb current densities as high as 2.5 $A\cdot m^{-2}$ regardless of the temperature, methane productivity is highly dependent on this parameter. In addition, and related to this, it seems that the electrotrophic process (represented by *Desulfomicrobium, Desulfovibrio, Sphingobacterium* and *Anaerolinea*) is more robust than the methanogenic process (represented by *Methanobacterium* and *Methanosaeta*), which translates into a higher reproducibility in current than in methane production. However, after 91 days of operation, the current also started to differ widely between the replicates. Although differences in relative abundance at the genera level in the biofilm composition might explain this divergent behaviour, other factors such as biofilm stratification or colonisation patters cannot be ruled out and might be worth investigating in future studies. Microbiological, CV and CO_2 cut-off tests confirmed the hypothesis of the two-step process and highlight the resilience of both the electrotrophic and the methanogenic communities to medium–low temperature conditions.

CRediT authorship contribution statement

Guillermo Peñaz: Conceptualization, Investigation, Methodology, Writing - original draft. **Daniela Carrillo-Peña:** Investigation, Methodology. **Antonio Morán:** Supervision, Writing - reviewing and editing, Funding acquisition, Project administration. **Adrián Escapa:** Conceptualization, Supervision, Formal analysis, Writing - reviewing and editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] Caizán-Juanarena L, Borsje C, Sleutels T, Yntema D, Santoro C, Ieropoulos I, et al. Combination of bioelectrochemical systems and electrochemical capacitors: Principles, analysis and opportunities. Biotechnol Adv 2020;39:107456. https://doi.org/10.1016/j.biotechadv.2019.107456.
[2] Bond DR, Lovley DR. Electricity production by Geobacter sulfurreducens attached to electrodes. Appl Environ Microbiol 2003;69(3):1548–55. https://doi.org/10.1128/AEM.69.3.1548-1555.2003.
[3] Kim HJ, Hyun MS, Chang IS, Kim BH. A Microbial Fuel Cell Type Lactate Biosensor Using a Metal-Reducing Bacterium. Shewanella putrefaciens. J Microbiol Biotechnol 1999;9:365–7.
[4] Li X, Ding L, Yuan H, Li X, Zhu Y. Identification of potential electrotrophic microbial community in paddy soils by enrichment of microbial electrolysis cell biocathodes. J Environ Sci (China) 2020;87:411–20. https://doi.org/10.1016/j.jes.2019.07.016.
[5] Jeremiasse AW, Hamelers HVM, Buisman CJN. Microbial electrolysis cell with a microbial biocathode. Bioelectrochemistry 2010;78(1):39–43. https://doi.org/10.1016/j.bioelechem.2009.05.005.
[6] Rabaey K, Read ST, Clauwaert P, Freguia S, Bond PL, Blackall LL, et al. Cathodic oxygen reduction catalyzed by bacteria in microbial fuel cells. ISME J 2008;2(5):519–27. https://doi.org/10.1038/ismej.2008.1.
[7] Zhu C, Wang H, Yan Q, He R, Zhang G. Enhanced denitrification at biocathode facilitated with biohydrogen production in a three-chambered bioelectrochemical system (BES) reactor. Chem Eng J 2017;312:360–6. https://doi.org/10.1016/j.cej.2016.11.152.
[8] Cheng S, Xing D, Call DF, Logan BE. Direct biological conversion of electrical current into methane by electromethanogenesis. Environ Sci Technol 2009;43(10):3953–8. https://doi.org/10.1021/es803531g.
[9] Sarker S, Lamb JJ, Hjelme DR, Lien KM. Overview of recent progress towards in-situ biogas upgradation techniques. Fuel 2018;226:686–97. https://doi.org/10.1016/j.fuel.2018.04.021.
[10] Li S, Song YE, Baek J, Im HS, Sakuntala M, Kim M, et al. Bioelectrosynthetic Conversion of CO2 Using Different Redox Mediators: Electron and Carbon Balances in a Bioelectrochemical System. Energies 2020;13(10):2572. https://doi.org/10.3390/en13102572.
[11] Cordas CM, Moura JJG, Escapa A, Mateos R. Carbon Dioxide Utilization—Bioelectrochemical Approaches. In: Enzym. Solving Humankind's Probl. Springer International Publishing; 2021. p. 83–108. https://doi.org/10.1007/978-3-030-58315-6_3.
[12] Bian B, Bajracharya S, Xu J, Pant D, Saikaly PE. Microbial electrosynthesis from CO2: Challenges, opportunities and perspectives in the context of circular bioeconomy. Bioresour Technol 2020;302:122863. https://doi.org/10.1016/j.biortech.2020.122863.
[13] Yang H-Y, Bao B-L, Liu J, Qin Y, Wang Y-R, Su K-Z, et al. Temperature dependence of bioelectrochemical CO2 conversion and methane production with a mixed-culture biocathode. Bioelectrochemistry 2018;119:180–8. https://doi.org/10.1016/j.bioelechem.2017.10.002.
[14] Lee SY, Oh Y-K, Lee S, Fitriana HN, Moon M, Kim M-S, et al. Recent developments and key barriers to microbial CO2 electrobiorefinery. Bioresour Technol 2021;320:124350. https://doi.org/10.1016/j.biortech.2020.124350.
[15] Blasco-Gómez R, Batlle-Vilanova P, Villano M, Balaguer M, Colprim J, Puig S. On the edge of research and technological application: A critical review of electromethanogenesis. Int J Mol Sci 2017;18(4):874. https://doi.org/10.3390/ijms18040874.
[16] Mateos R, Escapa A, San-Martín MI, De Wever H, Sotres A, Pant D. Long-term open circuit microbial electrosynthesis system promotes methanogenesis. J Energy Chem 2020;41:3–6. https://doi.org/10.1016/j.jechem.2019.04.020.
[17] Rusmanis D, O'Shea R, Wall DM, Murphy JD. Biological hydrogen methanation systems – an overview of design and efficiency. Bioengineered 2019;10(1):604–34. https://doi.org/10.1080/21655979.2019.1684607.

[18] Figeac N, Trably E, Bernet N, Delgenès J-P, Escudié R. Temperature and Inoculum Origin Influence the Performance of Ex-Situ Biological Hydrogen Methanation. Molecules 2020;25:5665. https://doi.org/10.3390/MOLECULES25235665.

[19] Lavergne C, Aguilar-Muñoz P, Calle N, Thalasso F, Astorga-España MS, Sepulveda-Jauregui A, et al. Temperature differently affected methanogenic pathways and microbial communities in sub-Antarctic freshwater ecosystems. Environ Int 2021; 154:106575. https://doi.org/10.1016/j.envint.2021.106575.

[20] Wang Y, Guo WQ, Xing DF, Chang JS, Ren NQ. Hydrogen production using biocathode single-chamber microbial electrolysis cells fed by molasses wastewater at low temperature. Int J Hydrogen Energy 2014;39:19369–75. https://doi.org/10.1016/j.ijhydene.2014.07.071.

[21] Mateos R, Sotres A, Alonso RM, Escapa A, Morán A. Impact of the start-up process on the microbial communities in biocathodes for electrosynthesis. Bioelectrochemistry 2018;121:27–37. https://doi.org/10.1016/j.bioelechem.2018.01.002.

[22] Villano M, Aulenta F, Ciucci C, Ferri T, Giuliano A, Majone M. Bioelectrochemical reduction of CO2 to CH4 via direct and indirect extracellular electron transfer by a hydrogenophilic methanogenic culture. Bioresour Technol 2010;101(9):3085–90. https://doi.org/10.1016/j.biortech.2009.12.077.

[23] Van Eerten-Jansen MCAA, Veldhoen AB, Plugge CM, Stams AJM, Buisman CJN, Ter Heijne A. Microbial community analysis of a methane-producing biocathode in a bioelectrochemical system. Archaea 2013;2013:1–12. https://doi.org/10.1155/2013/481784.

[24] Alonso RM, Escapa A, Sotres A, Morán A. Integrating microbial electrochemical technologies with anaerobic digestion to accelerate propionate degradation. Fuel 2020;267:117158. https://doi.org/10.1016/j.fuel.2020.117158.

[25] Ter Heijne A, Geppert F, Sleutels THJA, Batlle-Vilanova P, Liu D, Puig S. Mixed culture biocathodes for production of hydrogen, methane, and carboxylates. In: Adv. Biochem. Eng. Biotechnol. Springer Science and Business Media Deutschland GmbH; 2019. p. 203–29. https://doi.org/10.1007/10_2017_15.

[26] Van Eerten-Jansen MCAA, Heijne AT, Buisman CJN, Hamelers HVM. Microbial electrolysis cells for production of methane from CO2: long-term performance and perspectives. Int J Energy Res 2012;36(6):809–19. https://doi.org/10.1002/er.v36.610.1002/er.1954.

[27] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7(5):335–6. https://doi.org/10.1038/nmeth.f.303.

[28] Reeder J, Knight R. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. Nat Methods 2010;7(9):668–9. https://doi.org/10.1038/nmeth0910-668b.

[29] Baek G, Kim J, Lee S, Lee C. Development of biocathode during repeated cycles of bioelectrochemical conversion of carbon dioxide to methane. Bioresour Technol 2017;241:1201–7. https://doi.org/10.1016/j.biortech.2017.06.125.

[30] Leicester DD. Energy recovery from a high strength waste stream using pilot-scale bioelectrochemical systems. School of Engineering Newcastle University; 2020.

[31] Rozendal RA, Jeremiasse AW, Hamelers HVM, Buisman CJN. Hydrogen production with a microbial biocathode. Environ Sci Technol 2008;42:629–34. https://doi.org/10.1021/es071720+.

[32] Cordas CM, Guerra LT, Xavier C, Moura JJG. Electroactive biofilms of sulphate reducing bacteria. Electrochim Acta 2008;54(1):29–34. https://doi.org/10.1016/j.electacta.2008.02.041.

[33] Yu L, Duan J, Zhao W, Huang Y, Hou B. Characteristics of hydrogen evolution and oxidation catalyzed by Desulfovibrio caledoniensis biofilm on pyrolytic graphite electrode. Electrochim Acta 2011;56(25):9041–7. https://doi.org/10.1016/j.electacta.2011.05.086.

[34] Batlle-Vilanova P, Puig S, Gonzalez-Olmos R, Vilajeliu-Pons A, Balaguer MD, Colprim J. Deciphering the electron transfer mechanisms for biogas upgrading to biomethane within a mixed culture biocathode. RSC Adv 2015;5(64):52243–51. https://doi.org/10.1039/C5RA09039C.

[35] Bo T, Zhu X, Zhang L, Tao Y, He X, Li D, et al. A new upgraded biogas production process: Coupling microbial electrolysis cell and anaerobic digestion in single-chamber, barrel-shape stainless steel reactor. Electrochem Commun 2014;45: 67–70. https://doi.org/10.1016/j.elecom.2014.05.026.

[36] Wenzel J, Fiset E, Batlle-Vilanova P, Cabezas A, Etchebehere C, Balaguer MD, et al. Microbial community pathways for the production of volatile fatty acids from CO2 and electricity. Front Energy Res 2018;6. https://doi.org/10.3389/fenrg.2018.0001510.3389/fenrg.2018.00015.s00110.3389/fenrg.2018.00015.s002.

[37] Mateos R, Sotres A, Alonso RM, Morán A, Escapa A. Enhanced CO2 Conversion to Acetate through Microbial Electrosynthesis (MES) by Continuous Headspace Gas Recirculation. Energies 2019;12:3297. https://doi.org/10.3390/en12173297.

[38] Liu W, Cai W, Guo Z, Wang L, Yang C, Varrone C, et al. Microbial electrolysis contribution to anaerobic digestion of waste activated sludge, leading to accelerated methane production. Renew Energy 2016;91:334–9. https://doi.org/10.1016/j.renene.2016.01.082.

[39] Jiang X, Shen J, Lou S, Mu Y, Wang N, Han W, et al. Comprehensive comparison of bacterial communities in a membrane-free bioelectrochemical system for removing different mononitrophenols from wastewater. Bioresour Technol 2016;216: 645–52. https://doi.org/10.1016/j.biortech.2016.06.005.

[40] Rozendal RA, Hamelers HVM, Rabaey K, Keller J, Buisman CJN. Towards practical implementation of bioelectrochemical wastewater treatment. Trends Biotechnol 2008;26(8):450–9. https://doi.org/10.1016/j.tibtech.2008.04.008.

[41] Dykstra CM, Pavlostathis SG. Methanogenic Biocathode Microbial Community Development and the Role of Bacteria. Environ Sci Technol 2017;51(9):5306–16. https://doi.org/10.1021/acs.est.6b0411210.1021/acs.est.6b04112.s001.

[42] Zhou H, Xing D, Xu M, Su Y, Zhang Y. Biogas upgrading and energy storage via electromethanogenesis using intact anaerobic granular sludge as biocathode. Appl Energy 2020;269:115101. https://doi.org/10.1016/j.apenergy.2020.115101.