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INSTITUTO DE MEDIO AMBIENTE, RECURSOS NATURALES Y BIODIVERSIDAD

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EXPLORING CHALLENGES IN MICROBIAL ELECTROMETHANOGENESIS.

EFFECT OF POWER INTERRUPTIONS, TEMPERATURE, pH AND GAS COMPOSITION.

PROGRAMA DE DOCTORADO EN CIENCIA Y TECNOLOGÍA DEL MEDIO AMBIENTE

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ABSTRACT

Renewable energy and carbon capture and utilisation technologies have experienced a rise in recent years as a result of increased awareness of fossil fuel consumption and associated pollution. In this context, biogas from thermal process or anaerobic digestion has become a critical technology to simultaneously achieve waste management and bioenergy production. To meet natural gas specifications, the CH_4 content of biogas must be upgraded. Conventional biogas upgrading technologies use separation and sorption techniques, and although they are mature and applicable technologies, they are generally energy intensive. Bioelectrochemical systems (BES) have recently emerged as an alternative to these traditional biogas upgrading systems.

By means of electromethanogenesis (EM) in the biocathode of a BES, the CO_2 fraction of the biogas can be directly reduced to CH_4 in a biocathode, using surplus energy produced with renewable energies. However, this technology is still in an early stage of development and suffers from several challenges such as the intermittency of the power source, the influence of temperature, the influence of pH and the pollutants present in the biogas to be upgraded. In this context, the main objective of this thesis will be to investigate the influence that these factors have on EM, to perform a preliminary study on EM variability, understand their impact on the electrotrophic hydrogenogenic and methanogenic stages and to explore the technical feasibility of using a real biogas as feedstock.

The need to accommodate fluctuations intrinsic to renewable energy (mainly solar and wind) requires an understanding of the impact this power inconstancy would have on EM. This thesis explores the impact of 24 to 96 h

power outages on EM reactors to determine their effect on methane production rates, current density consumption, current conversion efficiency, and on the microbial communities that compose the cathode biofilm. During the power outages, the cathodes were operated with and without external H_2 supplementation to determine how the power outages affect the hydrogenogenic and methanogenic pathways. EM was resilient to power fluctuations, although process efficiency decreased in the absence of H_2 supplementation.

Another important aspect of EM is the effect that medium-low temperatures have on the electrotrophic and methanogenic stages. To address this issue, EM reactors were subjected to different temperatures (between 30 and 15 °C). Decreasing the temperature affected the methane richness of the product. Methanogenesis, rather than hydrogenesis, was affected and proved to be the main source of variability in EM.

Selectivity is another challenge faced by EM systems. It mainly dependns on the microbial communities that finally grow on the cathode and our hypothesis is that pH could play a key role. This thesis studies the impact of pH on the EM process both during start-up and during normal operating conditions. The acidic environment allowed a faster onset of methane production, and dropping pH improved performance up to pH of 4.5. Results also seemed to indicate that high local pH on the surface of the cathode prevented severe physiological disruptions on the microbial communities caused by low bulk pH.

The last challenge to be addressed in this thesis is the use of real biogas. The CO_2-rich off-gas phase from hydrothermal carbonisation (HTC) was used as a real substrate for an EM system. The work demonstrated that off-gas HTC can

be used as raw material in an EM system although there is a decrease in methane production of up to 50% probably caused by the presence of CO.

*RESUMEN (*SPANISH*)*

Las energías renovables y las tecnologías de captura y utilización de carbono han experimentado un aumento en los últimos años como consecuencia de la mayor concienciación sobre el consumo de combustibles fósiles y la contaminación asociada. En este contexto, el biogás procedente del proceso térmico o de la digestión anaeróbica se ha convertido en una tecnología fundamental para lograr simultáneamente la gestión de residuos y la producción de bioenergía. Para cumplir las especificaciones del gas natural, es necesario mejorar el contenido de CH_4 del biogás. Las tecnologías convencionales de mejora del biogás utilizan técnicas de separación y sorción y, aunque son tecnologías maduras y aplicables, adolecen de ser intensivas en energía. Los sistemas bioelectroquímicos (BES) han surgido recientemente como alternativa a estos sistemas tradicionales de mejora del biogás.

Mediante la electrometanogénesis (EM) en el biocátodo de una BES, la fracción de CO_2 del biogás puede reducirse directamente a CH_4, utilizando la energía excedente producida con energías renovables. Sin embargo, esta tecnología se encuentra todavía en una fase temprana de desarrollo y adolece de varios problemas, como la intermitencia de la fuente de energía, la influencia de la temperatura, la influencia del pH y los contaminantes presentes en el biogás que se va a mejorar.

En este contexto, el objetivo principal de esta tesis será investigar la influencia que estos factores tienen sobre la EM, llevando a cabo un estudio preliminar sobre la variación de la EM, entender el impacto en las etapas

electrotróficas hidrogenogénicas y metanogénicas y explorar la viabilidad técnica de utilizar un biogás real como materia prima.

La necesidad de acomodar las fluctuaciones intrínsecas a las energías renovables (principalmente solar y eólica) requiere una comprensión del impacto que esta inconstancia de energía tendría en la EM. Esta tesis explora el impacto de los cortes de energía de 24 a 96 horas en los reactores de EM para determinar su efecto en las tasas de producción de metano, el consumo de densidad de corriente, la eficiencia de conversión de corriente y en las comunidades microbianas que componen la biopelícula del cátodo. Durante los cortes de energía, los cátodos fueron operados con y sin suplemento externo de H_2 para determinar cómo los cortes de energía afectan a las rutas hidrogenogénicas y metanogénicas. El proceso de EM fue resistente a las fluctuaciones de energía, aunque la eficiencia del proceso disminuyó en ausencia de suplemento de H_2.

Otro aspecto importante de la EM es el efecto que tienen las temperaturas medias-bajas en las etapas electrotrófica y metanogénica. Para abordar esta cuestión, se sometieron los reactores de EM a diferentes temperaturas (entre 30 y 15 °C). La disminución de la temperatura afectó a la riqueza en metano del producto. La metanogénesis, más que la hidrogénesis, se vio afectada y resultó ser la principal fuente de variabilidad en la EM.

La selectividad es otro de los retos a los que se enfrentan los sistemas de EM. Este aspecto depende principalmente de las comunidades microbianas que se seleccionan en el cátodo y nuestra hipótesis es que el pH podría jugar un papel clave. En esta tesis se estudia el impacto del pH en la EM tanto durante el arranque como en condiciones normales de funcionamiento. El entorno ácido permitió un inicio más rápido de la producción de metano, y el descenso del pH

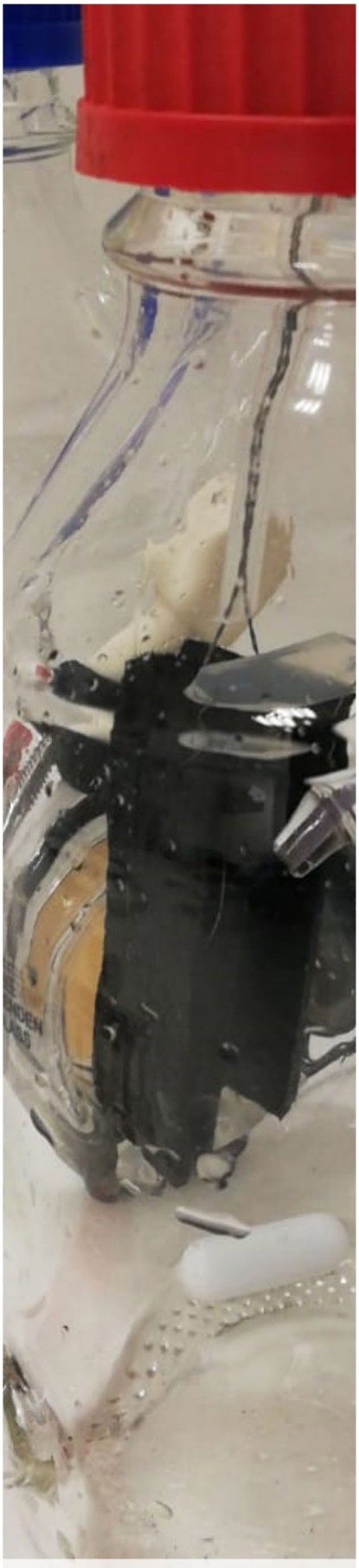
mejoró el rendimiento hasta un pH de 4,5. Los resultados también perecían indicar que un pH local elevado en la superficie del cátodo evitaba las graves alteraciones fisiológicas en las comunidades microbianas causadas por un pH global bajo.

El último reto que se aborda en esta tesis es el uso del biogás real. La fase off-gas rica en CO_2 procedente de la carbonización hidrotermal (HTC) se utilizó como sustrato real para un sistema de EM. El estudio demostró que el off-gas HTC puede ser utilizado como materia prima en un sistema de EM, aunque hay una disminución en la producción de metano de hasta el 50%. Este impacto fue mayor en la parte metanogénica del proceso, probablemente causado por la presencia de CO.

LIST OF ABBREVIATIONS

AD	Anaerobic digestion
Arc	Archaea
BES	Bioelectrochemical systems
CA	Chronoamperometry
CCS	Carbon capture and storage
CE	Coulombic efficiency
CEM	Cationic exchange membrane
CV	Cyclic voltammetry
DET	Direct electron transfer
DNA	Deoxyribonucleic acid
EM	Electromethanogenesis
E_w	Working electrode potential
FID	Flame ionisation detector
GHG	Greenhouse gas
HTC	Hydrothermal carbonization
I	Current
IC	Inorganic carbon
j	Current density
MEC	Microbial electrolysis cell
MES	Microbial electrosynthesis cell
MET	Microbial electrochemical technologies
MFC	Microbial fuel cell
n	Number of electrons
NOC	Normal operating conditions
OTU	Operational taxonomic unit
P2G	Power-to-gas
PI	Power interruption tests
PI+HS	Power interruption with hydrogen supplementation tests
Q_{AE}	Total electrical charge consumed by the abiotic electrode
Q_{BE}	Total electrical charge consumed by the biocathode
qPCR	Quantitative polymerase chain reaction
RE	Reference electrode
RNA	Ribonucleic acid

SD	Standard deviation
SHE	Standard hydrogen electrode
STP	Standard temperature and pressure
T	Temperature
t	Time
TB	Total bacteria
TCD	Thermal conductivity detector
TN	Total nitrogen
TOC	Total organic carbon
VFA	Volatile fatty acid
V_g	Amount of gas in bag
WE	Working electrode



CHAPTER 1

INTRODUCTION

1.1. Environmental and energy issues

Global population growth has brought an increase in the consumption of fossil fuels and raw materials [1], which results in pollution and greenhouse gas (GHG) emissions (specially CO_2). Under these circumstances, an economy based exclusively on the use of fossil fuels will have to face in the future energy sources depletion, environmental destruction, energy insecurity, social threats and economic instability [1–3]. As a consequence, technologies for renewable energy production have experienced an intense phase of research and development in recent years [1].

The main benefit of renewable energy sources is that they are readily available and constantly replenished, and can provide safe and sustainable energy and fuels. Renewable energies and materials are environmentally friendly, potentially without any environmental damage and support socio-economic development, secure supplies and climate change mitigation [1]. Renewable sources also contribute to alleviating the environmental and health impacts of human activity, because their use does not result in pollution or GHG emissions.

Thanks to a constant decrease in cost of equipment and installation, the share of renewable energies is expected to grow to 63% of total primary energy supply by 2050 [4]. Electricity is a common energy carrier from renewable sources, so renewable electricity production is expected to be abundant in the near future. However, renewable electricity is produced intermittently by most renewable sources. As a consequence, electricity needs to be stored when production is higher than demand and be supplied again when there is no or insufficient production.

1.2. Carbon capture and utilization

CO_2 emission reductions have become a necessity for industrialised countries due to the attention that global warming has been drawing [5–7]. However, as non-renewable fossil fuels remain the main source of energy, achieving CO_2 reductions is an elusive goal, even taking into account the significant advances in technological efficiency achieved in recent years. Carbon capture and storage (CCS) is a process capable of massively reducing anthropogenic CO_2 emissions into the atmosphere. It involves capturing waste CO_2 effluents from large-scale industrial sources and transporting them to a geological repository where the CO_2 is kept isolated from the atmosphere. However, there is scepticism about the high cost of CCS and there is growing public concern about underground CO_2 storage that has discouraged some demonstration projects in recent years [8,9].

In the past few years, CO_2 utilization has become an alternative to CO_2 mass storage [10], as CO_2 is an important precursor for several industrial process, and can be used as a propellant for fire fighting or as a supercritical solvent for sophisticated processes [11]. However, most of these applications require highly pure CO_2 which is difficult to obtain from intensive industrial processes such as fossil fuel combustion and from agricultural and livestock sites. In contrast, the conversion of CO_2 to chemicals or fuels represents an interesting alternative since the presence of impurities in the starting material is usually less problematic [12,13]. This could potentially close the carbon cycle in industry and the benefits from the sale of products could offset the costs of CO_2 capture [9,14].

CO_2 as a feedstock for chemicals and fuels started to receive attention after the oil embargo of the 1970s. Many organic and inorganic products can be obtained by electrocatalysis such as methane (CH_4), carbon monoxide (CO), ethylene (C_2H_4), methanol (CH_3OH) or formic acid (HCOOH) [15,16]. Electrocatalysis has the advantage that the formation of the different products is mainly dependent on the applied potential and the selected catalysts. However, the electrochemical conversion of CO_2 usually results in high overpotentials, low coulombic efficiencies, low kinetics or process stability and a limited product profile [17].

Since the carbon in CO_2 is fully oxidised, the reduction of CO_2 to organic products is an energy-demanding process [18–20]. To avoid the uncompetitive costs of using noble metals (gold, platinum, etc.) that have traditionally been used in electrochemical processes, biocatalysts can provide a cheap and ubiquitous alternative [9]. The microorganisms usually found in biocathodes possess the natural ability to activate CO_2 to produce mono- and multi-carbon organic products when they have access to reducing agents such as hydrogen, electrons from the cathode or other mediators [19,21].

1.3. Biogas and biogas upgrading technologies

Biogas is a mixture of gases mainly composed of CO_2 and CH_4, produced from the transformation of biowaste using thermal process or anaerobic digestion (AD). As a result, biogas production processes have become critical technologies to simultaneously achieve sustainable waste management and bioenergy production [22].

Among the thermochemical technologies for waste management, hydrothermal carbonization (HTC) represents a sustainable and cost-effective solution [23,24]. This process, that occurs under autogenous pressure and at moderate temperatures (150 °C - 300 °C) compared to conventional pyrolysis (400 °C - 600 °C), can convert organic wastes into different products [25,26]. The gaseous phase of pyrolysis, composed mainly of CO_2 (ca. 85-95%) with minor proportions of other gases such as CO, CH_4 or H_2 [26] and small traces of hydrocarbons [27] is commonly seen as a waste and only a few works have explored the possibilities of HTC off-gas conversion and valorisation [28,29].

AD is a complex biochemical process, that involves four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis [30]. The produced biogas is considered a clean and affordable renewable energy source to replace fossil fuels [31] but its composition (that typically consists of 60% CH_4 and 40% CO_2, with minor concentrations of other gases including H_2, NH_3, H_2S, and others [32]), brings important challenges in its utilization.

To meet the specifications of natural gas, the CH_4 content in biogas should be upgraded at least to 95%, at which point it is called biomethane or bionatural gas [33,34] and usually requires some kind of refining to improve its energy value

[35]. Conventional biogas upgrading technologies use separation or sorption techniques to reduce the CO_2 concentration of the raw biogas. There are four well-known technologies for CO_2 separation from biogas: water scrubbing, chemical scrubbing, membrane separation, and pressure swing absorption (PSA) [36,37]. Although these technologies are mature and applicable, they usually make an intensive use of energy [37,38].

Bioelectrochemical processes have recently emerged an alternative to these conventional biogas upgrading technologies, as they offer a highly sustainable route for CO_2 conversion to CH_4 and could potentially raise the CH_4 content of biogas above 90% [39].

1.4. Electromethanogenesis for biogas upgrading.

Bioelectrochemical systems are a family of devices that integrate microorganisms or other bio-based catalyst with electrochemical systems to enhance reduction and/or oxidation reactions [40,41]. BES have proven to be capable of catalysing a multitude of chemical reactions [42–47] (which will be addressed in more depth in the following chapter), among which electromethanogenesis (EM) outstands for its potential as an alternative to biogas upgrading.

Electromethanogenesis (EM) is a bioelectrochemical process where CO_2 is reduced to CH_4 in the biocathode [39,48]. EM can be considered a Power-to-Gas technology (P2G) with multiple potential practical applications, including wastewater treatment, GHG reduction, and renewable energy production [48–50].

EM could transform CO_2 from biogas into methane, increasing the value of the fuel while avoiding side streams [51], promising alternative for in situ biogas upgrading [52–54].

EM is still a lab-scale technology, but If we think of future practical-scale uses of EM, integration with anaerobic digestion is perhaps the most straightforward application [55]. Currently, two main alternatives have been proposed to carry out these integration: a) the implementation of electrodes inside a traditional digester, which requires fine control [56,57], and b) the use of BES as a post-treatment of AD, which greatly simplifies the operation of both systems and leads to a simpler set-up to optimise both processes.

Recent studies on the second strategy have successfully managed to keep the CO_2 content of a biogas below 10% while the carbon dioxide conversion efficiencies exceeded 80% [49,58]. Another study showed that biogas upgrading and energy storage via EM was possible using anaerobic sludge as biocathode [52]. Biocathodes were able to produce biogas with CH_4 content as high as 97.9 ± 2.3% at an applied voltage of 4 V and a gas flow rate of 17.79 mL/h. Despite these encouraging figures, and despite the great potential of EM for biogas upgrading, this technology still has a number of challenges that need to be considered before it can be applied outside the laboratory. Several of these challenges will be addressed in this thesis.

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CHAPTER 2

STATE OF THE ART

2.1. A brief history of bioelectrochemical systems: from power generation to fuel production

The history of BES began in 1911 when M.C. Potter discovered that microorganisms were capable of generating a potential difference in a galvanic cell [1]. However, although this discovery occurred more than 100 years ago, there was very little progress in this field during the 20th century. Only Cohen *et al.* revived such studies in the 1960s and 1970s in the context of NASA's space programme with the aim of cleaning water from human faeces and urine and harnessing it to generate electricity [2,3]. This was the first time that microbial electrochemistry had been considered technologically relevant and the archetype of electrochemical technology was born: the microbial fuel cell (MFC) (Figure 2.1) [4,5].

During the rest of the century the advances in BES were few. Perhaps the most important breakthrough was the discovery that by means of electron mediators the current density and power output could be increased [6]. However, it was not until the new millennium that the development of MFCs experienced an exponential growth. Based on the growing awareness of the depletion of fossil fuel resources and the environmental consequences of their use, worldwide scientific efforts focused on developing technologies for sustainable management of the environment and resources of our planet. This context allowed the revival of interest in MFCs [4].

In 2000, Kim *et al*. found that MFC operation using wastewater as a fuel could proceed without the need for artificial mediators facilitate electron transfer [7]. This discovery marked a turning point in the history of the BES because it

meant that microbes were able to transfer electrons directly to the anode [7,8], were operationally stable and could yield a high coulombic efficiency [5].

During the rest of the 00s, studies focused on microorganisms that were able to form a biofilm on the surface of the anode and were able to transfer electrons directly the anode electrode. These electroactive microorganisms included *Shewanella putrefaciens* [9], *Geobacteraceae sulferreducens* [10], *Geobacter metallireducens* [11] and *Rhodoferax ferrireducens* [8]. These studies contributed to the consolidation of MFCs as advanced wastewater treatment and power generation systems [5].

In addition, and during the first few years of this century, the anode was the main focus of the scientific development of BES (the cathode was mainly used as a counter-electrode). This changed when the cathodic reactions started to gain attention for the generation of energy-rich chemical compounds such as H_2, CH_4 and H_2O_2 [4]. Moreover, by the mid-first decade the reversibility of BES was demonstrated, which gave birth to the microbial electrolysis cells (MEC) (Figure 2.1). In 2005 Liu and Logan [12] published the first study on MEC technology, in which the use of electrochemically active microorganisms to break down organic substances at the anode was combined with an additional small voltage to produce H_2 at an abiotic cathode. MEC extended the applicability of BES by broadening the spectrum of compounds to be oxidised such as cellulose, glucose, glycerol, acetic acid, sewage sludge and complex wastewater [13,14].

The first reports on the use of the interaction of microorganisms and electrodes for chemical synthesis came when Hongo and Iwahara discovered that microbial fermentations can be directed by electrical current [15]. Their

experiments were later followed by Ghosh and Zeikus [16] as well as Emde and Schink [17], that formed the basis of current research in bio-electrosynthesis [5]. But it was not until 2007, when the first studies focusing on bio-mediated electrocatalysis at the cathode started to emerge with the discovery of suitable microorganism with ability to accept reducing equivalents from an electrode in pure cultures [18,19] or in consortia [20,21]. These pioneering studies demonstrated that by applying external electrical energy and using microorganisms as sustainable catalysts, biosynthesis applications could be developed to produce fuels (e.g. H_2 and CH_4) and high value-added chemicals such as acetate ethanol, butyrate, caproate, etc., [22–24] and highlighted the interplay between electron uptake and energy conservation as a critical constraint for cathodic biocatalysts [25].

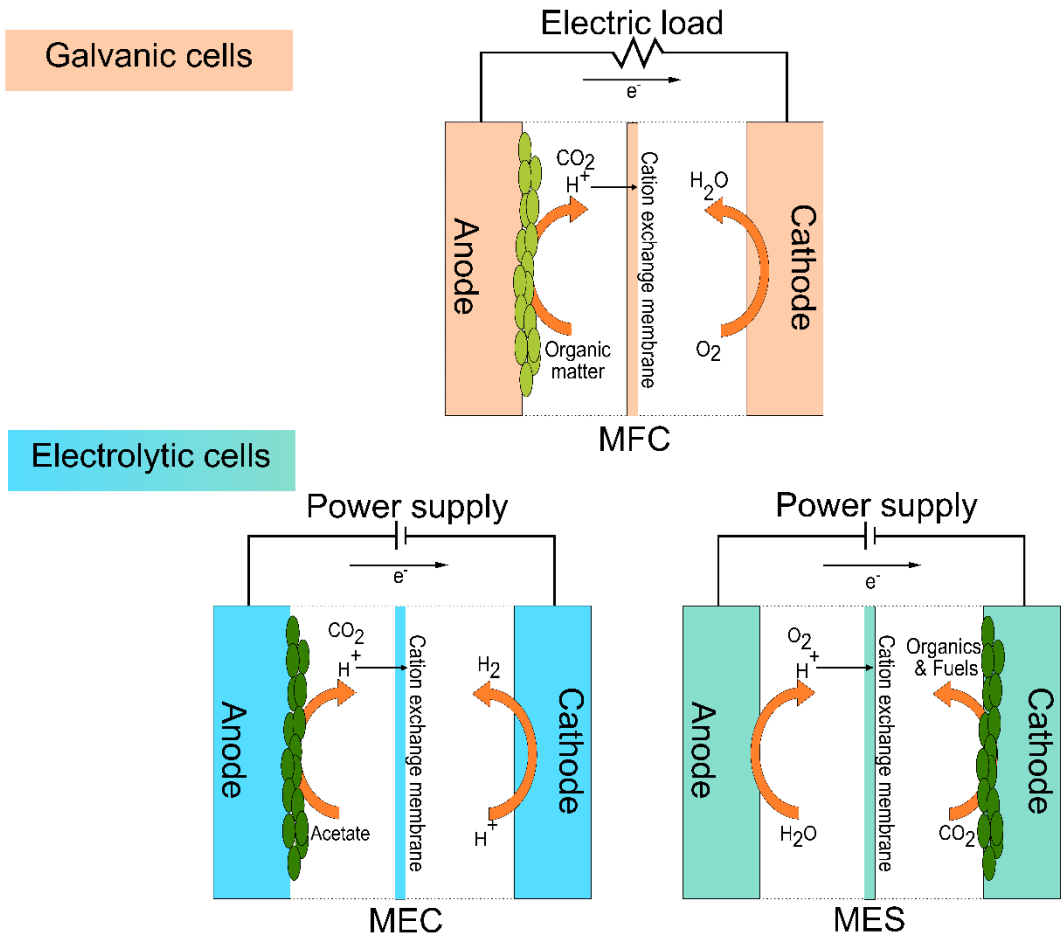

***Figure 2.1**. Diagrams of the main BES separated according to whether they are galvanostatically (MFC) or electrolytically (MEC and MES) operated.*

The term microbial electrosynthesis (MES) was first introduced in 2010 by Nevin *et al*. [23] who for the first time combined bioelectrochemical reactions with the synthesis of multicarbon compounds from CO_2 (Figure 2.1). This concept aroused great interest because of its potential to provide a direct pathway for CO_2 fixation and energy storage in chemicals. Since then, the microbial production of chemicals from carbon dioxide with electricity as an energy source has prompted numerous groups around the world to initiate research in the field of MES as it can be described in Figure 2.2 [22].

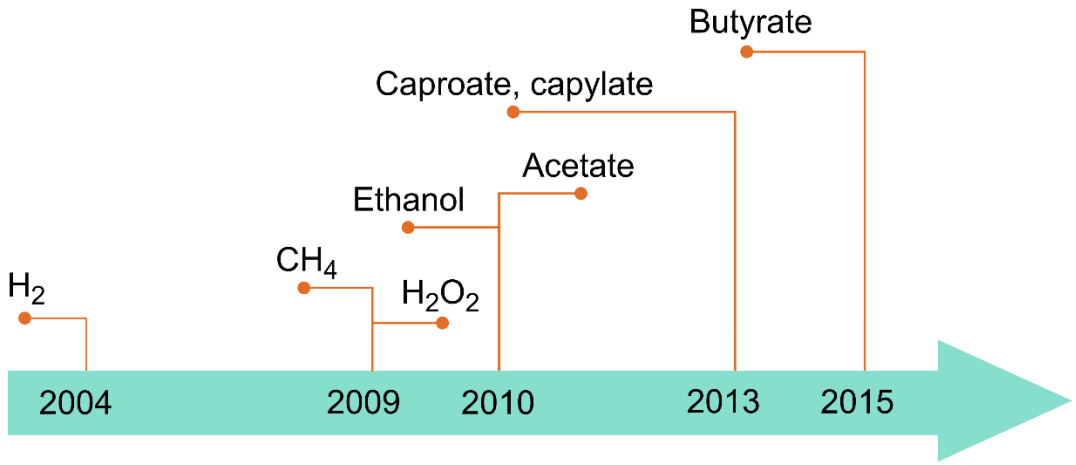

***Figure 2.2**. BES fuel and chemical discoveries* [22].

2.2. Fundamentals of MES

2.2.1. Architecture of MES

In brief, MES devices consist of several components: (i) an anode where an oxidation reaction takes places to provide electrons to the (ii) biocathode where organic products are produced by microorganisms, (iii) generally a separator, where positively charged ions migrate through from anodic chamber to the cathodic chamber to keep the solution electroneutral and (iv) and external source of electrical energy to drive the reactions [22,26].

The design and configuration play a key role in the performance of a MES. Single-chamber and double-chambers configurations are the two main reactor designs used in laboratory experiments. Single chamber MES have the advantage of simple configuration, no membrane cost and low input voltage due

to low internal ohmic resistance [27]. Although methane can occur in single-chamber configurations, as demonstrated by Qu *et al*., [28] and Bo *et al*., [29], there are limitations, especially related to the O_2 generated in the cathode, which can be harmful to oxygen-sensitive methanogens [30].

For biogas upgrading, two-chamber configurations usually represent a better choice: the process itself becomes more stable, and result in a higher CH_4 yield and significantly higher CH_4 content [31–33]. The separation of the two chambers involves the introduction of a separator or membrane. Its purpose is to create a physical barrier that prevents short circuits, oxygen and substrate crossover between the anode and cathode electrodes, while maintaining charge transfer between chambers.

Depending on the type of structural material, membranes are divided into two main categories organic, inorganic and mixed membranes [34]. The first type is based on polymers, e.g. Nafion or sulphonated polymers [35]. Although membranes based on natural polymers offer some unique characteristics, they can be susceptible not only to biofouling but also to deterioration [36,37]. In contrast, synthetic polymers such as expanded polystyrene can offer long-term durability, but also longer running response times. Inorganic separators consist mostly of ceramic separators, while mixed separators comprise composite membranes. Ceramic separators offer good energy efficiency but also high porosity, which can lead to high oxygen back-diffusion and substrate crossover, inducing biofouling effects [36].

The presence of the membrane inevitably leads to the occurrence of biofouling. Several strategies and monitoring approaches have been developed

to reduce bioadhesion to the separators, such as those based on blending with carbon materials, surface coating with polymers, doping with silver nanoparticles and the synthesis of new membranes with high antifouling potential and also physical and chemical methods to regenerate membranes already affected by biofouling [36,38,39].

Another key issue related to reactors configurations is the electrode material, which must be biocompatible, anti-corrosive, conductive, mechanically resistant, with a larger surface area and a surface morphology suitable for biofilm formation on the electrode surface [26,40]. There are two types of materials that are viable for use as electrodes: carbon-based or metal-based.

Carbon-based materials (such as graphite and carbon) are normally used as suitable cathode materials due to their high specific surface area, biocompatibility and chemical stability [33,41,42]. In contrast, metallic electrodes such as platinum, titanium or molybdenum are used as the counter electrode (anode) [26,43], because of their higher catalytic activity for the oxygen evolution reaction and stability.

2.2.2. Products of MES from CO_2

MES reactors had been typically fed with bicarbonate, synthetic mixtures of CO_2 and other CO_2-rich gases as a carbon source [44–47]. The inorganic carbon fed to a MES can be converted into different organic products, such as methane, volatile fatty acids (VFAs) and alcohols [48–51].

Acetic acid is the most common of the VFAs reported in MES, thanks to the versatility of homoacetogens [52], which can grow in a wide range of physicochemical conditions, usually involving CO, CO_2 and/or H_2 [53,54]. MES reactors also could produce butyrate, propionate, isobutyrate or even medium chain fatty acids which can be produced at moderately negative potentials or through chain elongation reactions [55–57]

Homoacetogens, responsible for acetate production, can also be producers of alcohols if sufficient protons or hydrogen are available [56,58]. Ethanol, butanol, isopropanol or glycerol have been reported to be produced in MES systems [33,54–56,59–62].

Methane is a common product of MES systems (when methanogens are present as biocatalyst) [63], where it can be produced through different routes: (i) via direct electron transfer mechanisms, (ii) via hydrogen-mediated mechanisms, and (iii) via acetoclasteic mechanism [64,65] (Table 2.1). This versatility allows methanogenesis to be more competitive than acetogenesis from a thermodynamic point of view [66].

The first study on methanogenesis in MES was carried out in a two-chamber bioelectrochemical system that produced approximately 4.5 $L \cdot d^{-1} \cdot m^{-2}$ methane with a coulombic efficiency of 80% [64]. Later studies were mainly devoted to explaining the mechanisms of bioelectrochemical methane production and to developing strategies to improve its production [67] allowing to reach 30.3 $L \cdot d^{-1} \cdot m^{-2}$ [68] with efficiencies ranging from 23% to 100% [69,70].

***Table 2.1**. Overview of methanogenesis and homoacetogenesis reactions by reduction of CO_2. Standar reaction potentials/energies were taken from Thauer et al.,* [71] *and expressed at pH 7, 298 K with all other reactants at standard concentrations* [66].

Process	Reaction	E'^0/V (vs. Ag/AgCl)
Half reactions		
Methanogenesis	$CO_2 + 8e^- + 8H^+ \rightleftharpoons CH_4 + 2H_2O$	−0.24 V
Acetogenesis	$2CO_2 + 8e^- + 8H^+ \rightleftharpoons CH_3COOH + 2H_2O$	−0.29 V
Hydrogen oxidation	$2H^+ + 2e^- \rightleftharpoons H_2$	−0.41 V
Overall reactions in anaerobic digestion		
Homoacetogenesis	$4H_2 + 2CO_2 \rightleftharpoons CH_3COOH + 2H_2O$	0.12 V
Hydrogenotrophic methanogenesis	$4H_2 + CO_2 \rightleftharpoons CH_4 + 2H_2O$	0.17 V
Acetoclastic methanogenesis	$CH_3COOH \rightleftharpoons CH_4 + CO_2$	0.05 V

2.2.3. Microbiology and energy transfer mechanisms

The microorganisms that populate the cathode (biocatalysts), are a key element in a MES. The presence of methanogens, hydrogenogens and acetogens are responsible for required end product.

The right inoculum is a decisive factor for the correct development of the MES reactor [72]. MES reactors can be inoculated with pure or mixed cultures. While the first studies in this field were mostly performed with pure cultures [19,23] most of the literature that subsequently dealt with CO_2 reduction in MES used mixed microbiological cultures as inoculums for the cathode chamber [73]. Natural mixed cultures have several advantages over pure cultures: (i) they are more flexible and resilient [74], (ii) can utilise a broad spectrum of low-cost

substrates, and (iii) generate possible higher production rates through synergistic effects [75,76]. In contrast, the production specificity of mixed cultures is lower compared to pure culture MES, which implies an obstacle for an industrial use for a single target production [74,77]

Typical sources of mixed cultures for MES reactors include anaerobic digester sludge from wastewater treatment plants, brewery wastewater, or stormwater pond sediments [32,78,79].

There are three main mechanisms of interspecies electron transfer (IET) by which micro-organisms can exchange electrons with electrodes: (1) the indirect electron transfer (IDET) mechanism uses soluble or gaseous substances to transfer electrons between microorganisms, (2) the direct interspecies electron transfer (DIET) mechanisms require physical contact between two species for electron transfer, and (3) the mediated electron transfer (MET) mechanism uses electron transport mediators that diffuse from mediator-producing to mediator-consuming cells to transport electrons [74,80].

In natural habitats and anaerobic digesters, IDET via hydrogen and formate are the dominant IDET mechanisms among syntrophic organisms [74]. The most common in MES is hydrogen since it can be produced biologically and abiotically depending on the potential used. Organisms related to hydrogen reduction mediation typically found in biocathodes are *Desulfovibrio* sp. and [81–84] or *Petrimonas* [85].

In DIET, the microorganisms take up electrons directly from the electrode surface, in the absence of any mediator. Many microorganisms have empirically proven to be able to perform this process although little information is known

about how electrons are actually acquired by them [86]. C-type cytochromes have been proposed to be responsible for electron transfer in organisms such as *Geobacter sulfurreducens*, *Sporomusa ovata* or *Shewanella oneidensis* [87,88]. However, this process has been also observed in species lacking cytochrome C-type such as *Clostridium ljungdahlii* [89].

There are a number of exogenous redox mediators (such as neutral red, methyl viologen, Fe(II), ammonia and others [59,90,91]) that have been shown to function as shuttles in DIET. Microorganisms such as *Pseudomonas* sp. or *Shewanella oneidensis* can produce their own mediators (flavins and phenazines [92,93]) to exchange electrons with the electrode. Mediators have the advantage that they can be used by microorganisms other than the producers, facilitating and boosting electron transfer, although reducing the efficiency of the process [90].

2.3. Current challenges of EM in MES

The production of methane through EM depends on factors such as reactor configuration [94], and on various operating parameters, such as cathode potential, pH, temperature, buffering capacity or the composition and concentration of the catholyte [95]. Furthermore, the coupling of MES to renewable energy sources will have to cope with the intermittency of production, which is a characteristic of this type of energy source, and microbial populations will have to deal with real substrates. For all the above reasons MES have not yet left the laboratory scale. Practical application of MES will require intensive R&D work to overcome the current economic and technological barriers. Here we

provide a quick survey of several of these factors, that will be addressed in the thesis.

2.3.1. Intermittency of power supply from renewable energy sources

In addition to periods of overproduction, renewables suffer from fluctuations and interruptions due to the unpredictability of sunlight and wind [96]. The resilience of the EM to intermittency in the power supply is key factor that needs to be understood before undertaking the scaling of this technology.

Previous studies on the impact of power supply interruptions on MES have focused on those systems dedicated to converting CO_2 and electricity into multi-carbon organic compounds. In this regard, a MES dedicated to produce acetate was subjected to absence of electricity for periods of up to 64 h, showing that the electro-autotrophic activity resumed once the power supply was restored [97]. Another study showed that after a long period (6 weeks) of power interruption of the power supply in a MES, acetogenic activity recovered after reconnection, although few days later methanogens became dominant and methane was the main product of CO_2 bioelectroreduction [98]. To date, no previous study has studied the impact of power interruptions on an EM system.

2.3.2. Temperature

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) [99] but also on its economics (i.e., the higher the temperature, the higher the energy requirements for heating).

Most of EM studies have used mesophilic operating temperatures (34-37 ºC) [95,100]. The possibility of performing EM at ambient temperature without the need for heating systems could lead to significant savings in the maintenance cost of an MES system. Previous studies have reported that EM is stopped below 10 ºC [101], which opens the possibility of operating EM reactors in the medium-low temperature range (between 10 and 30 ºC).

2.3.3. pH

pH is another critical operational parameter in MES. However, there is no agreement as to how pH may favour the different microbial communities [66]. pH around 7 has been found to be optimal for acetogenesis and chain elongation [102], while for most of the mesophilic methanogens, the optimal pH ranges from 6.8 to 8 and growth rate can be reduced considerably when pH is below 6.5 [30]. Therefore, MES reactors operated at neutral pH, methanogenic organisms can compete with acetogens for H_2, or even convert carboxylic acid into methane [103]. Both groups compete for electrons and/or hydrogen at the cathode and the pH of the medium could play a key role in the predominance of any of them during reactor start-up.

2.3.4. Real substrate

The raw material that provides the carbon source for the MES/EM process is another key factor. Lab-scale methanogenic biocathodes are usually fed with synthetic mixtures of CO_2 and other gases [44–46] or bicarbonate [26]. However,

from a future industrial application point of view, it is interesting to explore the technical feasibility of using a real CO_2-rich gas as raw material for EM. Previous studies indicate that anaerobic digestate biogas, despite containing potentially toxic gases such as H_2S and NH_3, can be used as a feedstock in EM for biogas upgrading [47]. However, there are other toxic compounds present in CO_2-rich effluents such as CO, whose impact still needs to be investigated in EM.

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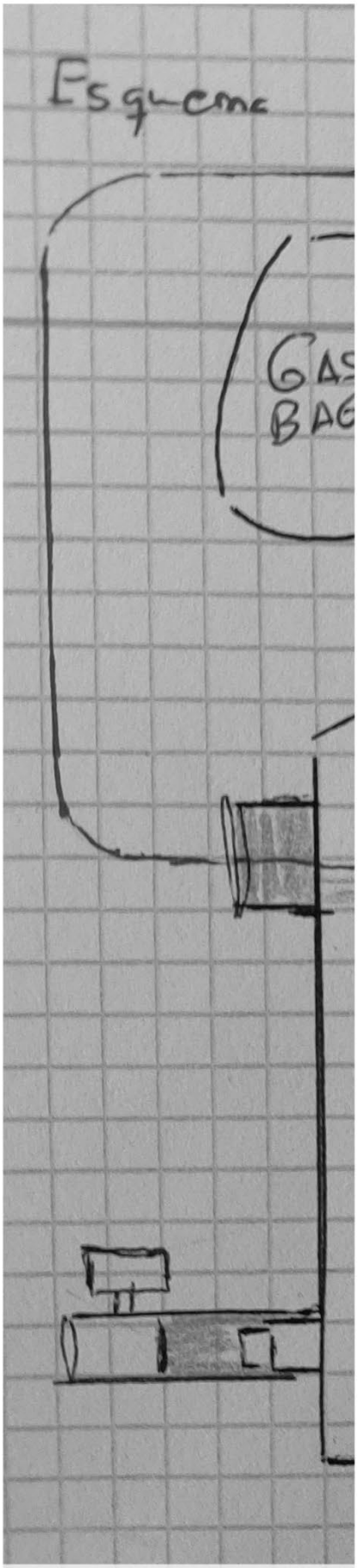

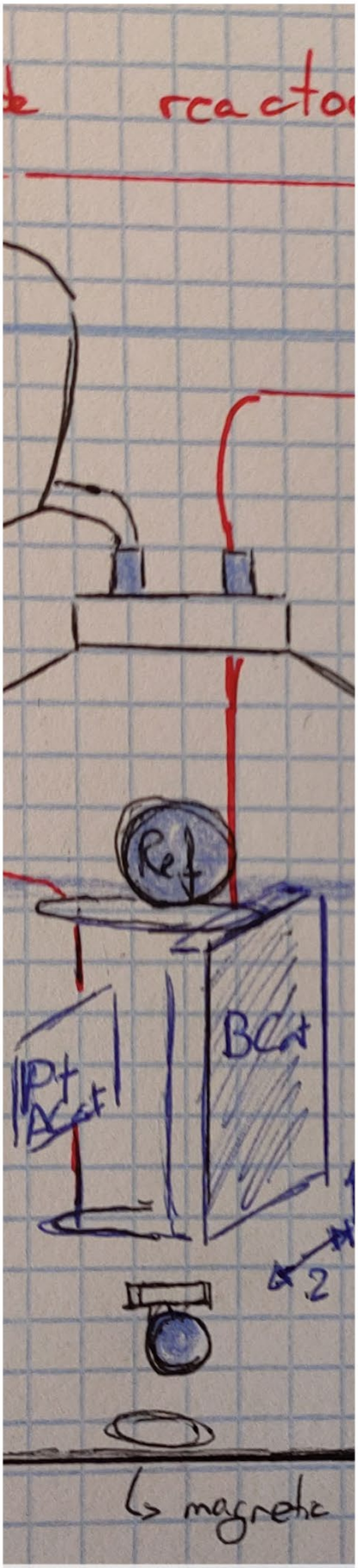

CHAPTER 3

OBJECTIVES AND THESIS OUTLINE

3.1. Objectives

The aim of this thesis is to study and understand several of the current limitations of the EM technology that stand in the way of its practical application. For this purpose, the following specific goals are proposed:

a) Study the impact of power fluctuations −typical of renewable energies− on the performance of an EM system.

b) Understand how temperature influences the two main metabolic stages of EM (i.e.: the electrotrophic and methanogenic stages) and its repercussion on the conversion rates and the quality of the produced gas.

c) Understand the role of pH in the selection of the cathodic microbial communities and their resilience to pH variations.

d) Explore the technical feasibility of using a real biogas as feedstock for EM, paying special attention to the effect of toxic gases on the processes and on the microbial communities at the cathode.

3.2. Thesis outline

Coupling surplus energy from renewable energy for CO_2 fixation and improving the quality of biogas entails a number of challenges which this thesis attempts to address. Firstly, the very unpredictable nature of renewable energy means that the EM must deal with the intermittency of renewable source. Other factors such as temperature and pH must also be addressed, as they play a key role in process performance and in the selction of the microorganisms that populate the cathode. Once this knowledge is consolidated, the next step should

be to evaluate this technology with real effluents in order to be able to compare the results with a view on future scale-up.

The present document is divided into 8 chapters (Figure 3.1):

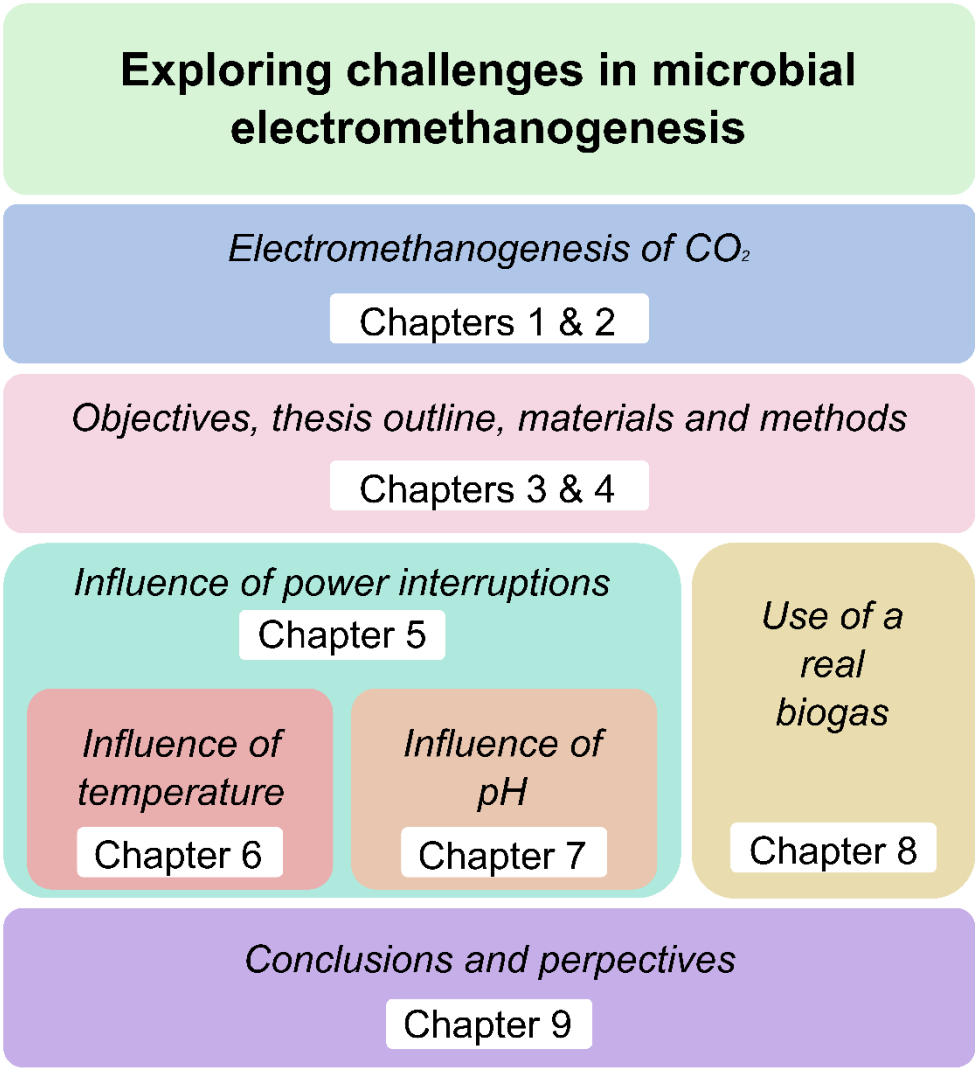

Figure 3.1. *General overview of this thesis*

Chapter 1. General introduction

This chapter provides a general context for the topics covered in this thesis, discussing those environmental and energy challenges that have led to the search for alternative energy conversion technologies such as EM.

Chapter 2. State of the art

This section briefly describes the history of bioelectrochemical systems up to electrosynthesis systems. It also includes the fundamentals of the bioelectrochemical systems and the current challenges of EM and MES technologies.

Chapter 3. Objectives and thesis outline

This part includes objectives, thesis outline, scope and structure of this thesis.

Chapter 4. Materials and methods

In this chapter, a brief description of materials and methods common to all chapters is given, including reactor design, electrolytes and inoculum, analytical methods and calculations, electrochemical and molecular biology techniques.

Chapter 5. Elucidating the impact of power interruptions on microbial electromethanogenesis

This chapter aims at gaining knowledge on how electromethanogenesis can potentially absorb the excess of renewable energy and store it as CH_4 despite the power fluctuations that these energy sources entail. In this research, power gaps from 24 to 96 h were applied to EM reactors to assess the effect of power interruptions on current density, methane production and current conversion efficiency. Furthermore, cathodes where operated with and without external H_2

supplementation during the power-off periods to evaluate how power outages affect hydrogenic and methanogenic steps separately.

Chapter 6. Impact of medium-low temperatures on electromethanogenesis

This chapter focuses on understanding de impact that medium-low temperatures have on electrotrophic hydrogenesis and hydrogenothrophic methanogenesis. This study evaluated the capability of EM to convert pure CO_2 into methane in a mild temperature range (from 30 ºC to 15 ºC), in terms of rates and quality of the biogas produced. This research included a preliminary effort to understand the sources of variability in EM.

Chapter 7. On the influence of pH on electromethanogenesis

This chapter tries to gain knowledge on pH as a decisive parameter in the selection of cathodic populations during start-up period. To achieve this, two MES reactors were started at pH 7 and pH 5.5 to assess competition between acetogens and methanogens. Furthermore, once the EM process was established, reactors were subjected to pH variations between 7.5 and 2.5 to study the impact on performance.

Chapter 8. Electromethanogenesis for the conversion of hydrothermal carbonization exhaust gases into methane

This chapter aims at gaining knowledge on how bioelectrochemical systems can potentially use a real off-gas to convert residual CO_2 into CH_4. To

achieve this, EM reactors were fed with the hydrothermal carbonization gas phase, a CO_2-rich gaseous phase that is commonly released directly into the atmosphere. Analysis focused on assessing CH_4 production and on the impact on the cathodic microbial communities.

Chapter 9. General conclusions and future perspectives

The general conclusions of this thesis are covered in this chapter. Moreover, some future perspectives on this technology are also included.

CHAPTER 4

MATERIALS AND METHODS

Although each of the experimental chapters in this thesis contains a section where the material and methods are described, this chapter provides a general description of reactors designs, inoculum and culture media, analytical and electrochemical techniques and calculations.

4.1. Reactors design and general operation

Experiments of chapters 5, 6, 7 and 8 were conducted in standard H-type reactors (Adams & Chittenden Scientific, CA, USA) with an internal volume of 500 mL per chamber. All reactor consisted of an anodic chamber and a cathodic chamber separated by a cation exchange membrane (CMI7000, Membranes International, USA). Membrane used to separate the anodic and cathodic compartments was pretreated in a 5% NaCl solution. The anode chamber was open to the atmosphere to prevent oxygen build up. The particularities of each reactor are described in detail in each chapter.

Graphite felt (SGL Group, Germany) was used as the biocathodes (working electrode) and platinum mesh (Goodfellow, UK) was used as anodes (counter-electrodes). Dimensions of electrodes are described in each chapter. Prior to inoculation, the graphite felts were pretreated by subsequent immersion in nitric acid 1 M, acetone 1 M and ethanol 1 M during 24 h each to avoid hydrophobicity and impurities [1].

The biocathodes were operated at a poised potential vs an Ag/AgCl reference electrode (Sigma-Aldrich, USA), using a Biologic VSP multichannel potentiostat and an EC-Lab software. Unless otherwise indicated, all the potentials will be referred to the Ag/AgCl electrode, whose stability was checked prior to every batch cycle.

Appropriate connections and sealings were designed for sampling ports and substrate supply. Gas was collected using a 1 L gas-bag (Ritter, Germany). A magnetic stirrer at 200 rpm was used in the catholyte in order to prevent the effect of mass transfer on current efficiency [2].

4.2. Electrolytes and inoculum.

Catholyte and anolyte of all experimental chapters consisted of 20 mM potassium phosphate buffer, macronutrients (280 $mg \cdot L^{-1}$ NH_4Cl, 5.7 $mg \cdot L^{-1}$ $CaCl_2$, 10 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, and 90 $mg \cdot L^{-1}$ $MgCl_2 \cdot 6H_2O$), 1 $mL \cdot L^{-1}$ of a micronutrients solution, and 1 $mL \cdot L^{-1}$ of a vitamin solution as described in [3]. As reactors were operated in batch mode, catholyte and anolyte were renewed at the beginning of each cycle.

For the experiments carried out in chapters 5, 6, 7 and 8, anaerobic sludge from a local wastewater treatment plant was used as primary inoculum. Inoculum was introduce in a 1:5 ratio of sludge to medium [4].

4.3. Analytical measurements and calculations

Liquid samples were analysed for total organic carbon (TOC) and total inorganic carbon (IC) and for volatile fatty acids (VFAs) from C2 to C6 (Bruker 450-GC) with a flame ionisation detector (FID). Dissolved oxygen (Hach, HQ40d - two-channel digit multimeter). 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 [1].

At the end of each batch cycle, the gas bag was emptied and the amount of gas in the bag (V_g) was measured with the aid of a gastight syringe (100 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) [1]. 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.

Cell performance was assessed in terms of coulombic (or current) efficiency (CE) (in %) and was calculated according to Eq. 1, where X is CH_4 produced (in mol), 8 mol e^- required to produce 1 mol CH_4, F is Faradaic constant (96485 C per mol e^-) and Q is the charged consumed (in C) [5].

$$CE = \frac{X*8*F}{Q} * 100 \quad \text{(Eq. 1)}$$

4.4. Electrochemical techniques

The electrochemical performance of the biocathodes was characterised by means of cyclic voltammetry (CV) tests using a Biologic VSP potentiostat at a temperature of 30 ºC. Voltage ranges and scan rates are specified in each chapter.

4.5. Molecular biology techniques

Microbial communities present on chapters 5 to 8 were analysed from samples taken from biocathodes (around 300 mg of each electrode), and bulk samples (300 mL) were collected and concentrated by centrifugation. These samples were used to characterise the microbial communities.

Genomic DNA was extracted with a DNeasy PowerSoil Kit (Qiagen) according to 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 and quantified by NanoDrop 1000 (Thermo Scientific).

The entire DNA extract was used for high-throughput sequencing of 16S rRNA gene-based massive libraries.16S rRNA gene-based primers for bacteria and archaea are specified in each chapter. Samples from chapter 5, 7 and 8 were analysed by Novogene Company (Cambridge, UK) carried Illumina sequencing out using a HiSeq 2500 PE250 platform and samples from chapter 6 were analysed by MrDNA Company (Shallowater, TX, USA), utilising MiSeq equipment (Illumina, San Diego, CT, USA).

The obtained DNA reads were compiled in FASTq files for further bioinformatics processing carried out using QIIME software version 1.7.0 [6]. Sequence analyses were performed by Uparse software (v7.0.1001) using all the effective tags. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database [7] for species annotation at each taxonomic rank (Threshold:0.8~1).

Quantitative-PCR (qPCR) analysis were performance to all samples of all experimental chapters by using PowerUp SYBR Green Master Mix (Applied Biosystems) in a StepOnePlus Real-Time PCR System (Applied Biosystems) as described previously [4]. The qPCR amplification was performed for the 16S-rRNA gene to quantify the total bacterial community and for the mcrA gene to quantify the total methanogen community. The primer sets 314F qPCR and 518R qPCR at an annealing temperature of 60 °C for 30 s was used for Bacteria and Arc 349F and Arc 806R for Archaea quantification.

4.6. References

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CHAPTER 5

ELUCIDATING THE IMPACT OF POWER INTERRUPTIONS ON MICROBIAL ELECTROMETHANOGENESIS

5.1. Introduction

According to the International Energy Agency, the growth of renewable power capacity will account for almost 95% of the increase in global capacity in 2026 [1]. To integrate these shares of variable electrical energy into the power system without compromising the grid stability and power quality, large amounts of energy storage capacity would be required. In this regard, and according to the same source, it is expected that energy storage will expand by 56% in 2026 (year 2021 as a basis).

Currently, there is a wide range of energy storage alternatives that, depending on their energy and power capacities, provide different benefits to the power grid [2]. Among them, power-to-gas (P2G) represents a developing energy storage technology capable of absorbing excess of renewable electricity and store it as hydrogen. Further combining this hydrogen with CO_2 to produce methane (either through chemical or biological methanation) can provide a more beneficial and flexible storage option [3,4], for it can be readily converted back into electricity (by means of a CHP unit), injected in the gas grid (as a carrier of renewable energy) or used as automotive fuel.

Bioelectrochemical methane production (also termed as Microbial electromethanogenesis (EM)) can be seen as a sub-branch of P2G. The distinctive feature of EM is that, thanks to the “catalytic” activity of a certain type of electroactive microorganisms, it allows direct conversion of CO_2 and electricity into methane in an engineered environment known as biocathode [5–7]. Unlike other CO_2 methanation alternatives, EM can occur without an external source of H_2, which avoids the need for hydrogen compression (an energy intensive

process) and the problems associated to hydrogen storage [8]. EM could thus provide in the future an environmentally-friendly, long-term energy storage solution to reduce the levelised cost of intermittent renewables [9]. EM also enjoys a fair degree of operational flexibility as it can find application in other sectors such as biogas upgrading or wastes treatment [10].

However, because of the relatively high capital costs of EM (chiefly explained by the cost of electrodes and membranes), and the low merit figures and efficiencies [11], EM has not yet entered the phase of commercial development. In addition, it is still uncertain how power interruptions, —derived from the intermittent and inconsistent nature of renewable power sources— will impact on the cathodic microbial communities and on the overall EM performance. Previous studies on microbial electrosynthesis systems (a bioelectrochemical technology similar to EM able to convert CO_2 and electricity into multicarbon organic compounds [12]) have revealed that the absence of electricity for periods of up to 64h affected production rates, although electro-autotrophic activity resumed once the power supply was restored [13]. In another study it was shown that after a long period (6 months) of power interruption in an acetate-producing microbial electrosynthesis reactor, acetogenic activity recovered after reconnection [14]. However, few days later, methanogens became dominant and methane was the main product of CO_2 bioelectroreduction.

Therefore, this chapter seeks to investigate the influence of power-off periods on the performance and microbial communities of an EM system. This study also aims at understanding how power outages impact on the two main metabolic stages of EM (i.e.: the hydrogenic and methanogenic steps), by supplementing externally-generated hydrogen during certain power-off periods.

The hydrogen supplementation tests allowed us to study the impact of power interruptions on hydrogenic and methanogenic steps independently.

5.2. Material and methods

5.2.1. Reactors

Standard H-type reactors (Adams & Chittenden Scientific, CA, USA) with an internal volume of 500 mL per chamber were used for the experiments. All reactor consisted of an anodic and a cathodic chamber separated by a cation exchange membrane (CMI7000, Membranes International, USA).

Two graphite felts (SGL Group, Germany) separated and kept in place by two silicon sheets were used as the biocathodes (working electrode). The total effective area of the biocathodes was 90 cm^2 (6 x 3 x 0.5 cm each of the two felts). In addition, a 2 x 2 cm platinum mesh electrode (Goodfellow, UK) was fixed under biocathode (separated 2 cm by a silicon sheet). The purpose of this additional electrode (cathode) was to provide hydrogen to the biocathode during the power-interruption periods in the hydrogen-supply experiments. Two 2 x 2 cm platinum mesh (Goodfellow, UK) were used as anodes (counter-electrodes).

Biocathodes were operated at a poised potential of -1.0 V vs an Ag/AgCl reference electrode (Sigma-Aldrich, USA), using a Biologic VSP multichannel potentiostat and an EC-Lab software. The platinum mesh cathodes were operated at a set current of 4 mA to generate abiotic H_2 when required. The biocathode and the platinum mesh cathode were connected to two independent

channels of the potentiostat (see Figure 5.1) through titanium wires covered with heat-shrinkable tubes to avoid unwanted electrochemical reactions.

Appropriate connections and sealings were designed for sampling ports and substrate supply as illustrated in Figure 5.1. Gas was collected using a 1 L gas-bag (Ritter, Germany). Reactors were kept inside a thermal chamber (Fitotron, SANYO) that maintained the temperature at 30 ± 0.5 °C. The catholytes were continuously stirred using a magnetic stirrer at 200 rpm in order to prevent mass transfer limitations [15].

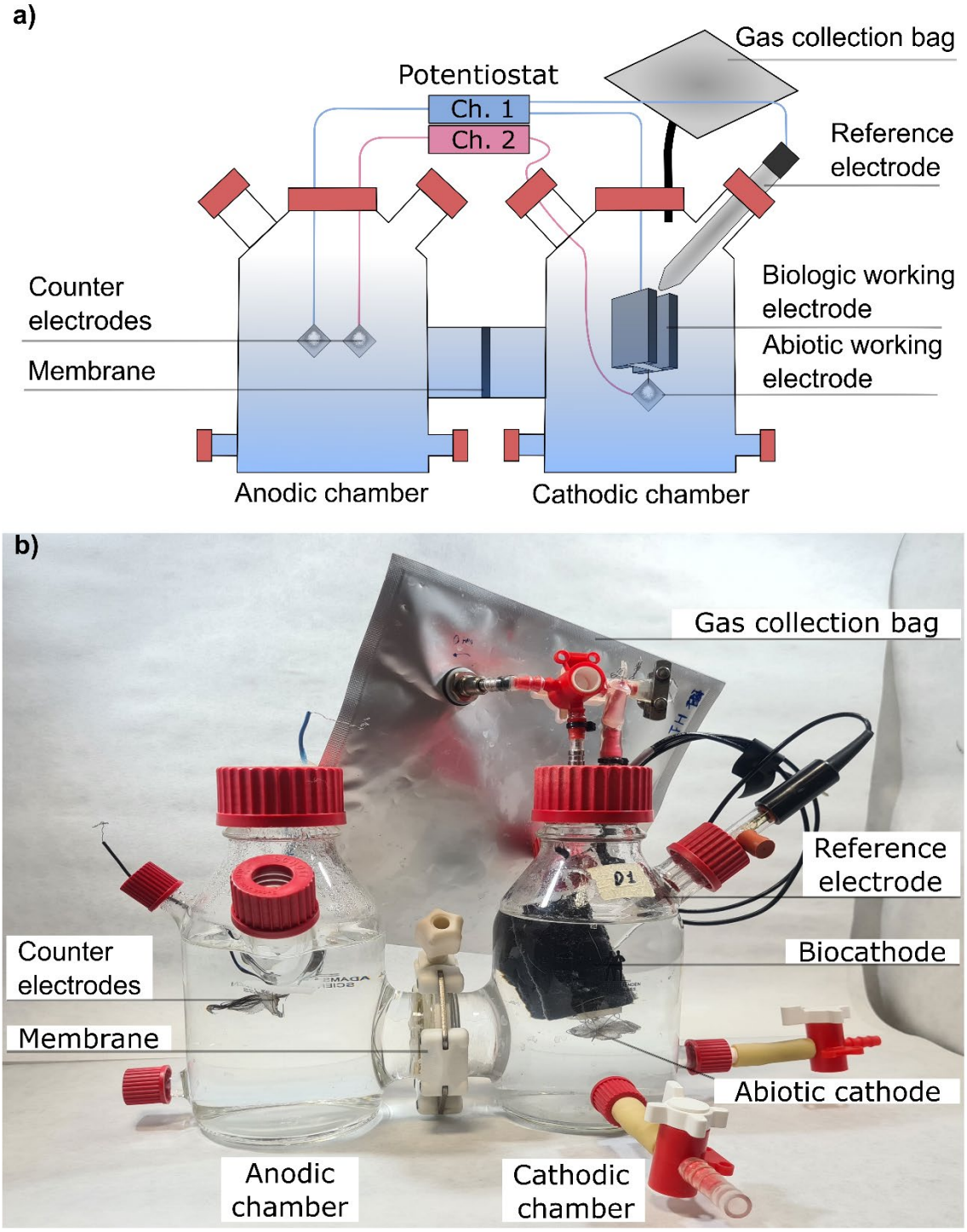

***Figure 5.1**. a) Schematic diagram of reactor; b) real image of reactor.*

5.2.2. Operation

In addition, 0.4 L of CO_2 (99.9% purity) were supplied as the only carbon source. After 16 consecutive cycles current density profiles recorded for both reactors tended to be repeatable between cycles. At that point the platinum mesh cathode was placed in the cathode chamber as explained before and the experimental phases began.

The experimentation was divided into 11 tests (numbered from 0 to 10 in Table 5.1). Each test condition was repeated in triplicate and they were randomly scheduled (allowing a rest period of 72 h between test in which the biocathodes were polarised at −1.0V) to avoid any habituation effect. The 11 tests were grouped into 3 set of tests with different aims and different operating conditions, as described below:

Power interruption with hydrogen supplementation tests (PI+HS)

In the course of the PI+HS tests (tests 1 to 4 in Table 5.1) the biocathodes were subject to power supply interruptions of 24 h, 48 h, 72 h and 96 h, that were programmed at the beginning of each cycle (the total duration of each cycle was 96 h). After that, the biocathodes were polarised at −1V for the remainder of the cycle. During the power interruption interval, hydrogen was supplied to the biocathode by means of the platinum mesh cathode that was operated at a constant current of 4 mA. This value was selected to be similar to the mean current produced by the biocathode when polarised at −1.0 V. With this, we sought that methanogens receive an almost similar amount of reducing equivalents during the entire tests regardless of the duration of the power gaps. In addition, and to prevent the formation of any biofilm on the platinum mesh

cathodes, they were immersed in a nitric acid 1 M solution before the beginning of each test.

Power interruption tests (PI)

In the course of the PI tests (tests 6 to 9) the biocathodes were subject to the same schedule of power interruptions, although no hydrogen was supplied during the power interruption intervals (i.e.: the abiotic cathode remained inactive). Following the power gap, the biocathodes were polarised at −1V in the same way as in the PI+HS tests.

Normal operating conditions (NOC)

During tests 0, 5 and 10 the biocathodes were operated in normal conditions, which means that they were polarised at −1.0 V vs Ag/AgCl during the 96 h duration of the cycles while the abiotic platinum cathodes remained inactive. These tests provided a baseline against which compare the performance of the reactors measured during the PI and PI+HS tests.

***Table 5. 1**. Summary of tests. All tests had a duration of 96 h. "Power interruption" indicates the number of hours in which the biocathodes remained disconnected from the power supply. "Hydrogen supplementation" indicates the number of hours along which the biocathodes were supplied with the hydrogen produced by the abiotic platinum cathode. "Electrodes polarization" refers to duration of the period during which either of the electrodes (biocathode or platinum) were active. It is the summation of the two previous columns "Power supply" indicates the number of hours along which the biocathodes were polarised. **NOC:** normal operating conditions. **PI+HS**: power interruption with hydrogen supply. **PI**: power interruption without hydrogen supply.*

Operating conditions	Tests number	Power interruption (h)	Hydrogen supplementation (h)	Electrodes polarization (h)
NOC	0	0	0	96
PI+HS	1	24	24	72
	2	48	48	48
	3	72	72	24
	4	96	96	0
NOC	5	0	0	96
PI	6	24	0	72
	7	48	0	48
	8	72	0	24
	9	96	0	0
NOC	10	0	0	96

5.2.3. Analytical measurements and calculations

TOC, IC, dissolved oxygen, redox and ammonium were analysed from liquid samples.

Gas composition was measured by gas chromatography. Methane production was normalized to the effective surface area of the biocathodes and was expressed in $L \cdot m^{-2}$. Methane production rate was calculated as the methane production divided by the duration of the period during which either of the two electrodes (biotic or abiotic) was active (the last column in Table 5.1). In short, this parameter intends to quantify the rate at which hydrogen (regardless of its biological or abiotic origin) is converted to methane.

5.2.4. Analytical measurements and calculations

CV tests were performed at the end of each cycle at a scan rate of 1 $mV \cdot s^{-1}$ and a voltage range between −1.0 V and 0.0 V at a temperature of 30 ºC.

5.2.5. Molecular biology techniques

At the end of test 0, 4, 8 and 9 (Table 5.1), small cathode samples were cut from biocathodes (around 300 mg of each electrode), and bulk samples (300 mL) were collected and concentrated by centrifugation. These samples were used to characterise the microbial communities.

DNA extracted was used for high-throughput sequencing of 16S rRNA gene-based massive libraries. 16 rRNA universal primers were V4 515F-806R for bacteria and V4 349F-806R for archaea. Quantification analysis of total bacteria and archaea was performed by qPCR.

5.3. Results and discussion

Following inoculation, the cathodic biofilms of reactors D1 and D2 were allowed a maturation period of 16 cycles (96 h duration per cycle). Throughout cycles 13 to 16 current density and methane production tended to stabilise, so it was assumed that biofilms were mature enough to start the experimental phase.

5.3.1. PI+HS tests

During the power-off periods with hydrogen supplementation tests, abiotically produced hydrogen was fed to the biocathodes. This set of experiments were intended to gain insight on the impact of power gaps on the hydrogenic step without compromising the methanogenic step.

Figure 5.2 shows that during PI+HS tests, averaged current density kept fairly stable (roughly 0.38 A m^{-2} in D1 and 0.33 A m^{-2} in D2) with no clear dependence on the duration of the power interruptions —excluding, of course, test 4 where the power supply was disconnected for the entire cycle—. Methane production rates followed a similar trend, and kept around 1.22 $L \cdot m^{-2} \cdot d^{-1}$ in D1 and 1.09 $L \cdot m^{-2} \cdot d^{-1}$ in D2 (Figure 5.2), regardless of the duration of the power interruptions. This result indicates that the methanogens proliferating on the cathodic biofilm can effectively use the externally added hydrogen during the power gaps, and that their metabolism is not dependent on the biocathode potential, thus confirming the role of hydrogen as a mediator in EM methane formation [6,16]. The contribution of planktonic communities to methane

production can be neglected as the catholyte (i.e.: growth medium) was completely renewed at the beginning of every test.

At the end of the PI+HS set of NOC tests were established again on both reactors (test 5). Current density values were similar to those measured during the power interruption tests, which indicates that the electrotrophic step is not affected by the power interruption. Methane production rates also resumed previous values, highlighting thus the resilience of the methanogens proliferating on the cathode biofilm to power interruptions.

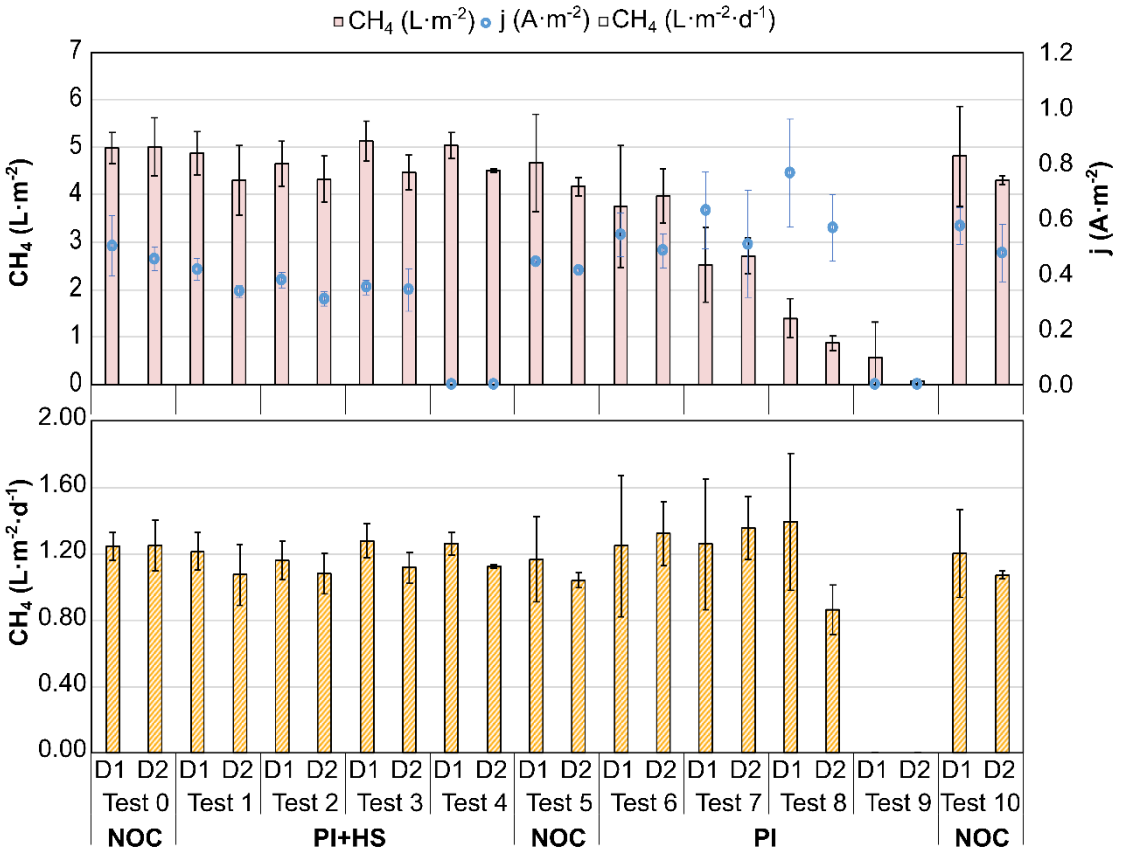

***Figure 5.2**. Above: net methane (in L per m² of electrode) and current density (in A·m^{-2}). Below: methane production rates (in L per m² of electrode and per day) for reactors D1 and D2 across the different tests.*

5.3.2. PI test

Interestingly, when reactors where subjected to the same program of power interruptions ─although with no hydrogen supplementation (PI tests in Table 5.1)─, current density values (see Figure 5.2) were significantly larger than those observed during the PI+HS tests for both D1 and D2 (p-values of 0.0031 and 0.0027 for D1 and D2 respectively). Taking a closer look at the current density profiles (Figure 5.3), important differences between the PI+HS and the PI tests can be observed. Typically, when a biocathode is re-connected after a power interruption, current increases sharply during the first few minutes, and then it tends to stabilise [13]. In these experiments, this phenomenon was only visible in the PI tests, and it became more pronounced the longer the duration of the power interruptions. Contrastingly, this trend was totally reversed during the PI+HS tests, where the size of the initial peak current decreased with the duration of the power gap.

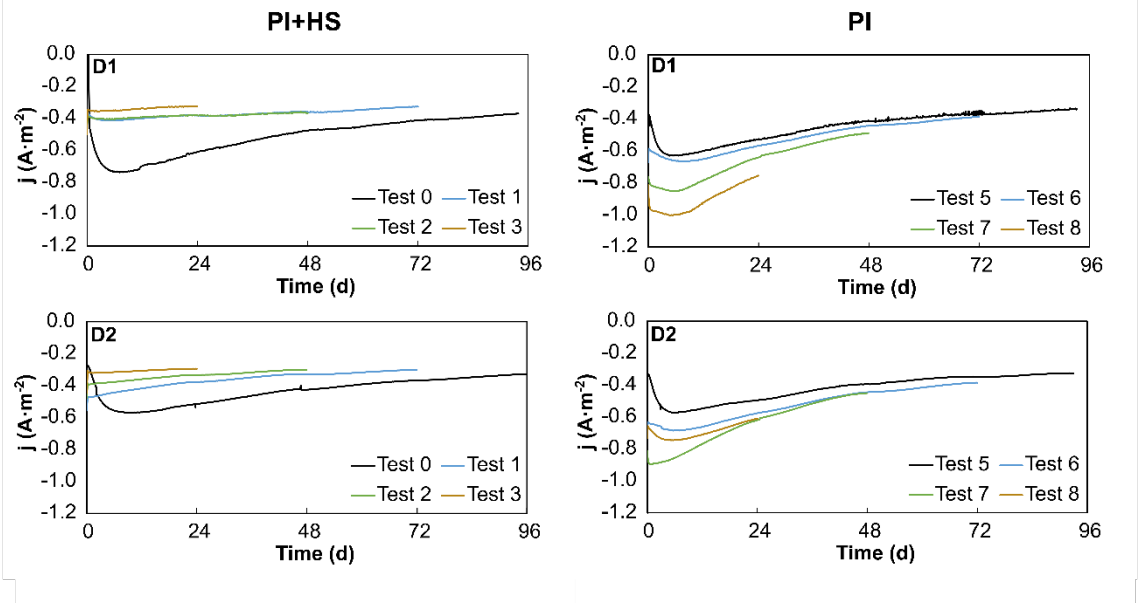

***Figure 5.3**. Typical current density profiles recorded for the different tests (for clarity reasons, only one current profile out of the three replicates was selected for each test). To facilitate the comparation between tests, all the profiles are represented starting at time 0. However, as explained in section 5.2.2, that the biocathodes were polarised right after the power interruption; so, for instance test 3 in PI+HS and 8 in PI would really begin at the hour 72.*

This finding can most probably be linked to the presence/absence of externally added hydrogen on the cathode chamber (see Figure S5.1 in Appendix 1). Indeed, if we assume that during the power gaps hydrogen partial pressure regulates the intracellular redox state of the electroactive hydrogenic bacteria —as it happens for instance with fermentative hydrogen producing bacteria [17,18]— then, in the absence of hydrogen (PI tests), the oxidised form of the redox cofactor inside the cells would tend to accumulate. As a result, once the biocathode is polarised again after the power gap, larger currents will be demanded by the electroactive bacteria to rebalance their redox state. In addition,

and following the same logic, when hydrogen is externally added (PI+HS tests), the reduced form of the redox cofactor would predominate, and consequently lower current would be demanded by bacteria after reconnection.

This hypothesis would be partially supported by the research published in [19], where the addition of NAD+ (the oxidised form of a common redox cofactor) to a hydrogen-producing biocathode increased both current density and hydrogen production. However, when the reduced form (NADH) was supplemented, no impact was observed on current nor on hydrogen production.

As expected, net methane production declined with duration of the power interruption during the PI tests (Figure 5.2). However, no significant differences were observed between the production rates with and without hydrogen supplementation (p-values of 0.4394 and 0.2729 for D1 and D2 respectively), which indicates that the methanogenic activity is resilient to power interruptions of up to 72 h even in the absence of hydrogen. However, it is important to note that both, current and methane production rate were less reproducible without hydrogen supplementation as evidenced by the larger error bars (see Figure 5.2). In addition, current was less efficiently used in the production of methane during the PI tests as evidenced by the coulombic efficiency (CE) values. CE, that computed as the ratio between the Coulombs actually transferred to methane and the total of coulombs (whether of biotic or abiotic origin) entering the cathode chamber, was significantly lower when there was no external hydrogen supplementation (p-values of 0.0007 and 0.0256 for D1 and D2 respectively). Indeed, whereas CE kept around 90% during the PI+HS tests (Figure 5.4), it seemed to decline with the duration of the power-off periods along the PI tests (from around 90 % with no power interruption to 40-60% for the 72 h of power

interruption). The lack of any electron donor on the cathode chamber (either in the form of electrons or externally added hydrogen) for long periods of time might be inducing a metabolic shift –or even a change in the populations–, that would explain the large variability in current and methane production and also the decline in CE. The slight decrease of the pH, together with the presence of significant amounts of TOC in the catholyte at the end of the PI tests (Figure 5.5), would support any of the two possibilities. It is also important to note that the qPCR analysis, as it will be shown in the next section, revealed a notable decline in both bacteria and archaea after the PI experiments.

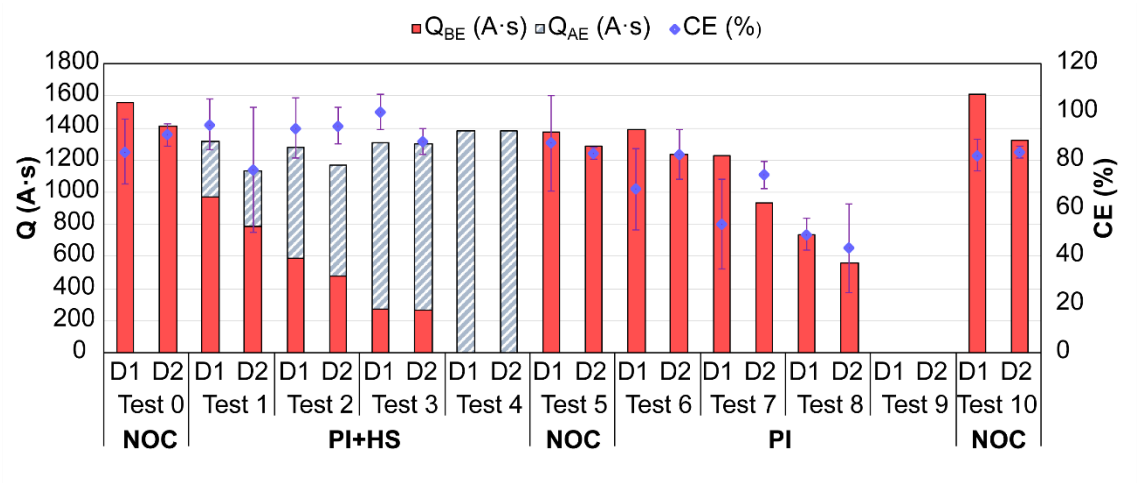

***Figure 5.4**. Columns represent the total electrical charge (in A·s) consumed by the biocathode (Q_{BE}) and the abiotic platinum electrode (Q_{PE}). The blue squares represent the coulombic efficiency.*

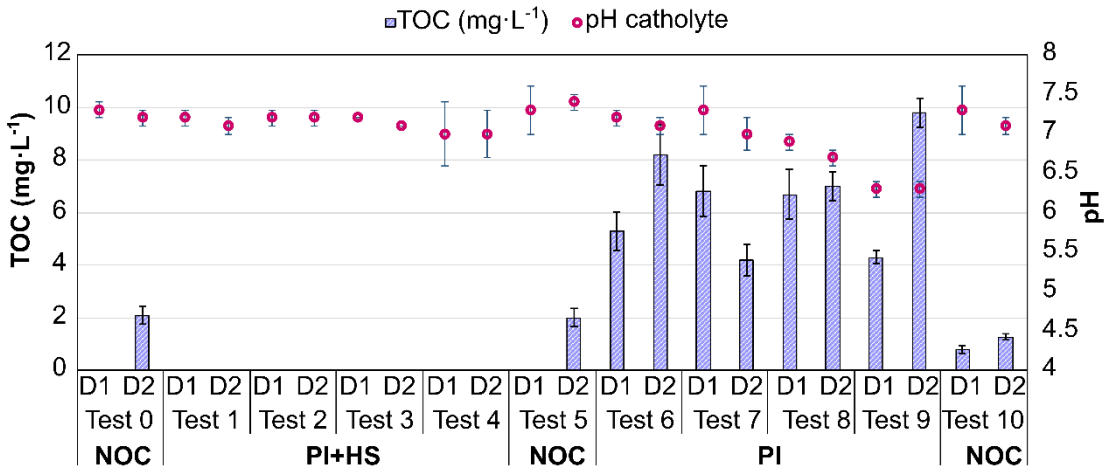

Figure 5.5. *Total organic carbon (TOC) in mg·L^{-1} and pH in the catholytes.*

Finally, and despite the frequent interruptions of the power supply during the PI tests, the reactors recovered their previous performance when normal operating conditions were imposed again (test 10). No significant differences were observed between the values of the current density, methane production rate and coulombic efficiency measured in Tests 5 and 10 (beginning and end of the PI tests respectively).

5.3.3. Cyclic voltammetry tests

Cyclic voltammetry tests (CV) were conducted at the end of each test (see Figure 5.6). The voltammograms recorded during the PI+HS experiments showed how the catalytic wave associated to hydrogen evolution (i.e.: potentials bellow −0.9V [20–22]) contracted as the length of the power interruption increased. Contrastingly, when there was no externally added hydrogen (PI tests), the length of the tails increased with the length of the interruption period. This

result is coherent with the significantly higher current densities measured during the PI tests, and supports the hypothesis that the presence of externally added hydrogen limits the activity of the eletrothrophic hydrogens.

When the biocathodes were disconnect for the entire cycle (96 h) in the presence of hydrogen supplementation (test 4 in PI+HS), the resulting voltammogram underwent a large transformation compared to those recorded under shorter power gaps. The most prominent feature was perhaps the appearance of a large reductive wave starting at −0.5V that might be linked to acetate production [23]. This peak also appeared ─though less apparent─ at large disconnection periods (72-96 h) in the absence of hydrogen supplementation (PI experiments). Another peculiarity of the 96 h-disconnect voltammograms was the total disappearance of the oxidation peak that occurred between -0.8V to -0.6V that has been linked to H_2 oxidation [23]. All these evidences suggest a shift in the metabolism of the hydrogen producing bacteria induced by long inactivity as argued in the previous section. Nevertheless, this shift was temporary and reversible, because when biocathodes were polarised again at −1V for the entire cycle (96 h), the voltammograms adopted a shape similar to those recorded during the power interruption experiments.

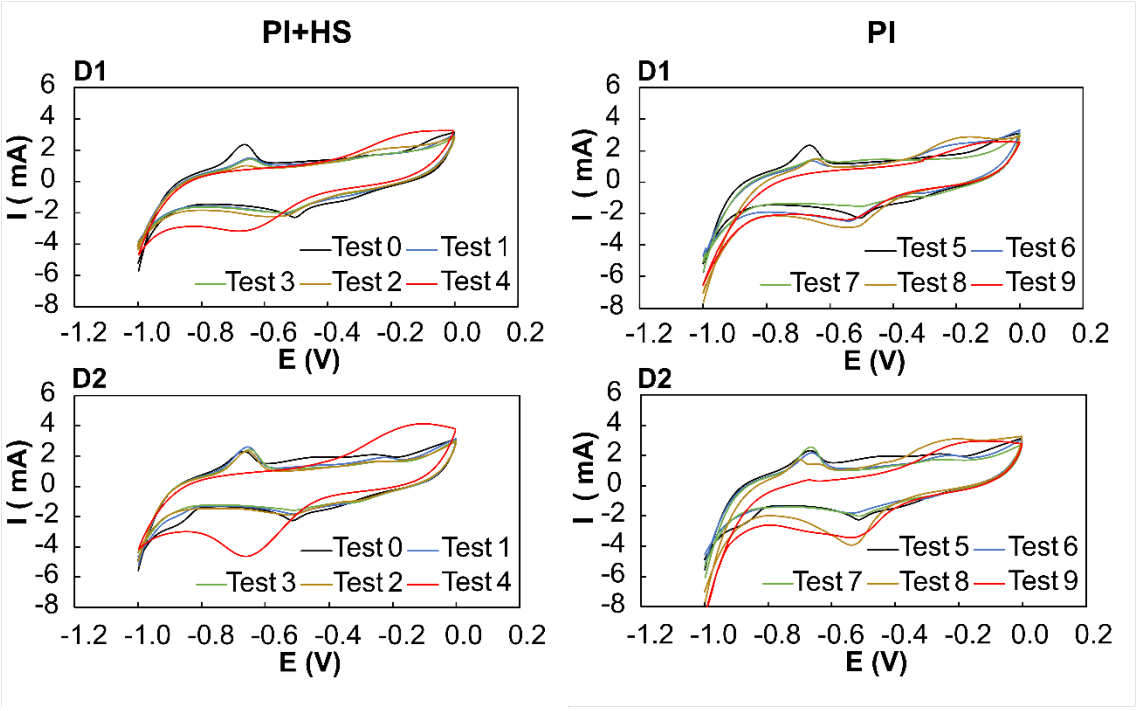

Figure 5.6. *Cyclic voltammograms for D1 and D2 during the PI+HS and PI set of tests. For clarity reasons, only one voltamogram for each power interruption period and set of experiments is shown.*

5.3.4. Microbiology

Before commencing the power interruption tests, the biofilms population was dominated by bacteria (around 70% bacteria vs. 30% archaea), although the single most abundant genus in both reactors was the archaea *Methanobacterium* (29 and 28% for D1 and D2 respectively) (Figure 8).

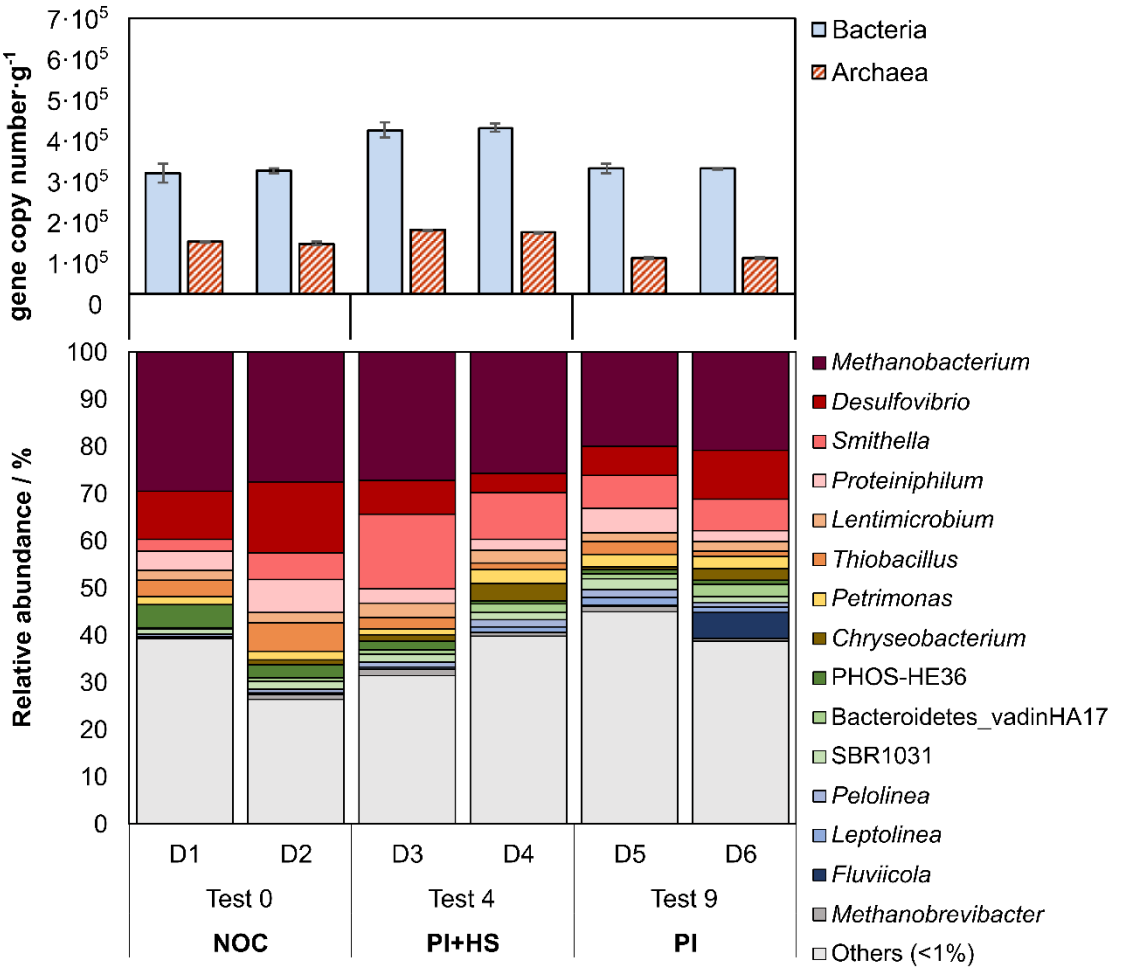

***Figure 5.7**. Above: qPCR analyses for reactors D1 and D2 at the end of tests 0, 4 and 9 in terms of Bacteria or Archaea. Below: Relative abundance for Reactors D1 and D2 during test 0 (initial conditions), test 4 (last 96 h outage with H_2 supplementation), test 9 (last 96 h outage without H_2 supplementation).*

Methanobacterium species live on the reduction of CO_2 with H_2 to produce CH_4, which is coherent with the hypothesis that H_2 is the main intermediary in the electron transfer between the electrodes and CO_2 [6,7,24,25]. Organisms catalysing hydrogen production such as *Desulfovibrio* (10% and 15% for D1 and D2) [23,26,27] or *Petrimonas* (2% and 2% for D1 and D2) [28] would confirm the syntrophic relationship between the electron uptake of the biocathode and the

final methane production. Another relevant organism found was *Lentimicrobium*, whose species are able to produce acetate, propionate and hydrogen, and experiences syntrophic growth with hydrogenotrophic methanogens [29].

Biofilm samples taken at the end of the PI+HS set of tests (test 4) showed a marked growth in both bacterial (around 40%) and archaeal populations (around 27%) (Figure 5.7). The presence of externally added hydrogen would obviously account for the archaeal increase, as most of the archaeal genus found in our reactors are hydrogenotrophic. However, the bacterial growth is more difficult to clarify. *Desulfovibrio*, which was the most important bacterial genus before the PI+HS tests underwent a sharp decline in its relative abundance, from 10% to 4% in D1 and from 15% to 7% in D2. This genus includes electroactive hydrogen-producing species, and its decline can be justified by the presence of externally added hydrogen, being also coherent with the lower current density measured during the PI+HS tests as discussed in sections 5.3.1. Therefore, if hydrogen affected negatively some bacteria, it must have also created a niche opportunity for other bacteria if we are to account for the overall bacterial growth. Interestingly, the *Smithella* species ─which are propionate-oxidizing syntrophic bacteria─ experienced a notable growth (from 3% to 10% in D1 and from 6% to 16% in D2). Therefore, the cathodic biofilm must have supported the development of other bacteria that produce the metabolites required for their proliferation. The large catalytic wave detected at −0.5 V in CVs at the 96 h disconnection tests (Figure 5.6) would support this conjecture. The absence of any organic carbon in the catholytes during PI+HS test (Figure 5.5), might be revealing a fast-local consumption of this metabolites in the biofilms.

The PI set of tests resulted in a large decrease of archaea (around 40% decline at the end of test 9) that in in our reactors are represented by the genus *Methanobacterium.* It seems then that the hydorgenotrophic methanogens, which are at the top of the methanogenic syntrophic community, are very sensitive to a prolonged lack of any reducing agent (either hydrogen or electrons) in the cathode chamber. Bacterial communities also suffered a significant decline (around 20%), although the main hydrogenogenic electrotrophic group (*Desulfovibrio*) showed an increase in relative abundance (6% for D1 and 10% for D2), which again is in accordance with the larger current density observed during the PI tests, and that might be related to the absence of externally added hydrogen, which favoured its metabolism as discussed before. It is important to note that only during the PI tests TOC was consistently detected in the catholytes (Figure 5.5), so it seems that the loss of activity of hydrogenotrophic methanogens may have induced a metabolic shift that contributed to the formation of other products such as VFAs.

5.4. Conclusions

This chapter investigates the effect of electrical power interruptions on the performance of a microbial electromethanogenic system. Results indicate that power gaps of up to 96 h do not have a significant impact of the methane production rates regardless of the supplementation of hydrogen. However, when these power gaps occurred in the absence of externally added hydrogen, current was less efficiently used in the production of methane. In addition, both current density and methane production rate became less reproducible (large variations

between replicates), which can be attributed to a disruption in the metabolic routes or a shift in the microbial populations caused by a prolonged lack of electron donors. Overall, this study shows that EM can stand relatively large periods of power interruption without a significant impact on performance

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CHAPTER 6

IMPACT OF MEDIUM-LOW TEMPERATURES ON MICROBIAL ELECTROMETHANOGENESIS

Abstract

In this chapter we try to understand the impact that medium-low temperatures have on the two main steps that usually comprise the electromethanogenesis (EM) process: electothrophic hydrogenesis and hydrogenothrophic methanogeneis. Results revealed that pure CO_2 can be effectively converted into a high purity biogas (~90:10 CH_4/CO_2) at 30 °C. However, when temperature is reduced, methane richness greatly decreases. This deterioration in performance was mostly attributed to a decline in the methanogenic activity (mainly represented by *Methanobacterium* and *Methanobrevibacter)*. In contrasts, the hydrogenic activity (mostly *Desulfomicrobium)* did not suffer any significant decay. Results also seem to indicate that methanogenesis, rather than hydrogenesis, is the main source of variability in EM. Rising the temperature again to 30 ºC returned previous performance, which highlights the resilience of EM after wide temperature fluctuations (30-15-30 ºC).

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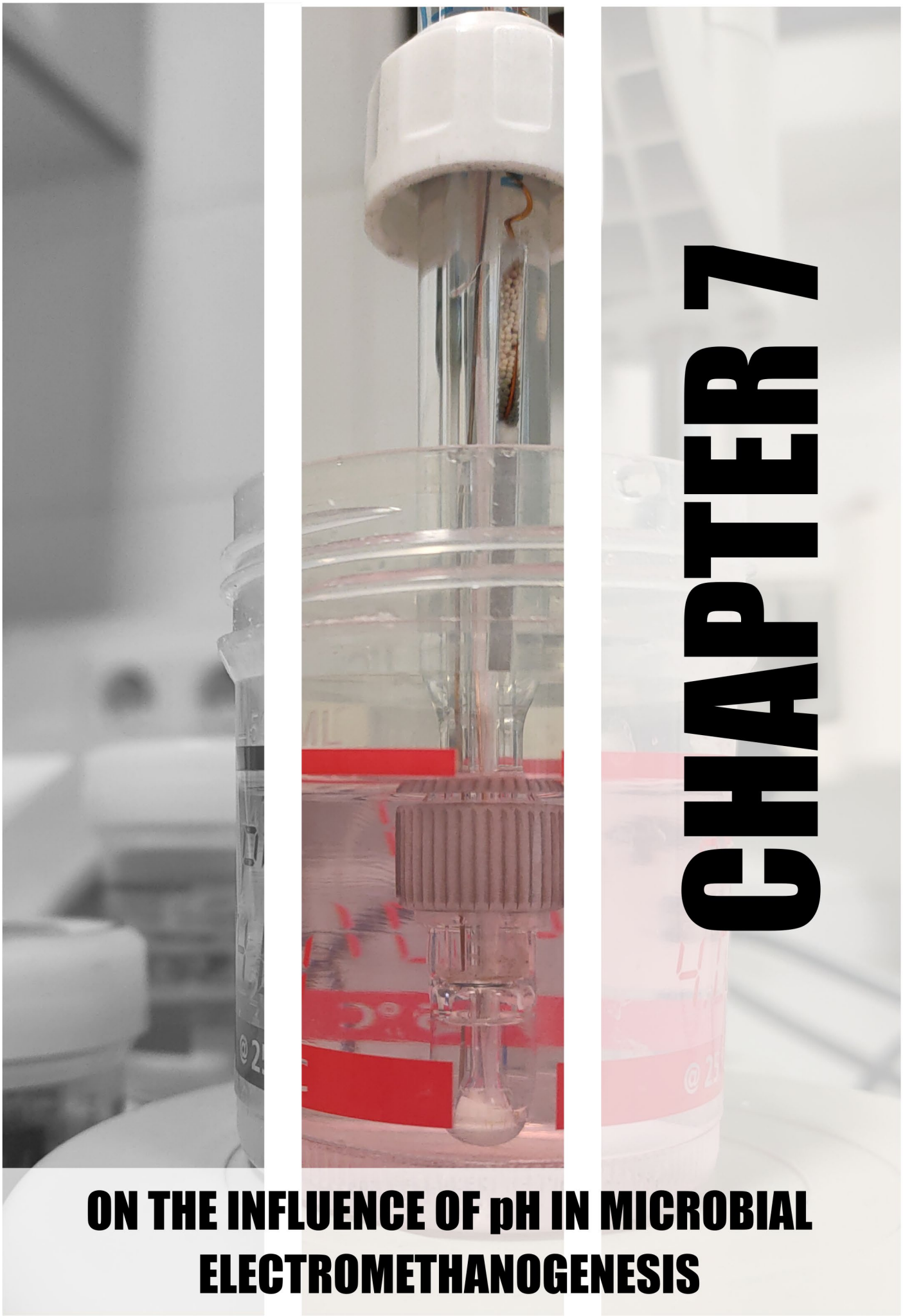

CHAPTER 7

ON THE INFLUENCE OF pH IN MICROBIAL ELECTROMETHANOGENESIS

7.1. Introduction

The decarbonisation of the energy and chemical sectors, which has become a global priority, has fuelled the research and development of Microbial electrosynthesis (MES), a technology that has emerged in the recent years as an alternative to more conventional organic synthesis processes [1].

MES is based on the ability of certain microorganisms (usually referred to as electrotrophs [2]) to catalyse the electrochemical reduction of CO_2 to a wide range of organic compounds [3–6]. When the end product is methane, MES is usually termed as biological electromethanogenesis (EM). EM has attracted a considerable interest among scientists and engineers because of its notable advantages in comparison to conventional thermoelectrocatalytic methanogenesis [7,8]. For instance, EM can be carried out at ambient (temperature and pressure) conditions using microorganisms as catalysts, all of which potentially makes EM a more environmentally friendly and cost-effective way of methane production. However, important technological constraints stand in the way of the development of MES and EM to a practical scale [9]. One of them is the low selectivity of MES, which mainly depends on reactor configuration [10], and on various operating parameters such as cathode potential, pH, temperature, buffering capacity or the composition and concentration of catholyte [11]. Among them, pH plays a key role as it has a direct impact not only on the process itself but also on the selection of the microorganisms that will populate the biofilm. When mixed cultures are used as inoculum, methanogens and homoacetogens can compete for H_2 [1], a common intermediate usually found in biocathodes for the conversion of CO_2 to both methane and carboxylates [12]. It is known that most mesophilic methanogens have an optimal growth pH in the

range between 6.5 and 8 [13] and that their methanogenic activity is inhibited when the pH drops below 3.8-4.5 [14,15]. In contrast, homoacetogens prefer mild acidic to neutral pH and are inhibited at high alkaline conditions [16]. Acetogenesis and chain elongation in particular have an optimal pH range between 7 and 5 [17,18]. Reducing the pH from 7 to 5.8 has already been used in a continuous acetate MES reactor fed with CO_2 to increase substrate availability and enhance production rate [19].

However, little is known about the influence of pH on the start-up of a MES/EM system and on the process performance. The starting hypothesis used in this study, based on the available literature, is that an acidic pH (of around 5.5) might be suitable for acetogenic activity, while a neutral pH (about 7) would provide a more favourable environment for the development of methanogenic microorganism. Therefore, in this study we try first to understand the influence that pH has on product selectivity and on product formation during the inoculation and start-up period. We also investigate the effect of pH on EM performance and the resilience of cathodophilic communities to pH modifications.

7.2. Materials and methods

7.2.1. Reactor design

Two standard H-type reactors were used with an internal volume of 500 mL per chamber named R7 and P5. The biocathodes were made with two 2 x 6 cm carbon felt plates (SGL Group, Germany) attached by titanium wire and suspended inside the cathodic chamber with a graphite rod (surface area: 0.0064 m²). The counter electrodes were made with 2 x 2 cm platinum mesh (Goodfellow, UK) suspended inside the anodic chamber with titanium wire (Figure 7.1).

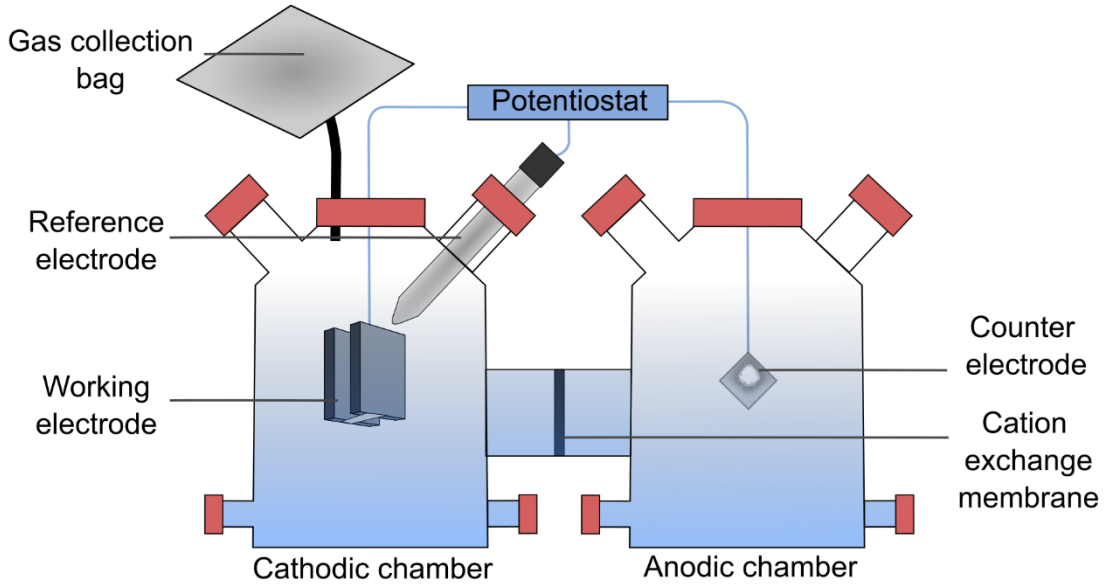

Figure 7.1. *Reactor diagram: right chamber contains a platinum mesh anode and is open to air and left chamber contains a carbon felt cathode, bag to store gases, reference electrode and connections for sampling and bubbling N_2 between cycles.*

Reactors worked on a three-electrode configuration using an Ag/AgCl commercial reference electrode (Sigma-Aldrich, USA). Data acquisition and −1 V

potential was carried out with a potentiostat (Biologic VSP) and EC-Lab® software (ver. 11.31).

Reactors temperature were stored in a constant temperature chamber (30º).

7.2.2. Operation

The influence of pH was determined at 2 different stages: during start-up and during normal operating conditions.

7.2.2.1. Operation during start-up

Reactors were inoculated with anaerobic sludge from a wastewater treatment plant (1:5 proportion) as it has been described before [20] and were operated in batch-mode in cycles of 5 days duration, fully replenishing electrolytes and gas composition at the beginning of each cycle.

One of the reactors (it will be referred to as P7) was inoculated and operated at pH 7, while the other reactor (referred to as P5) was inoculated and operated at pH 5.5.

7.2.2.2. Operation after start-up

After the start-up, and once the reactors achieved a stable performance, the pH in reactor P5 was modified according the program presented in Table 7.1. For each condition, 3 replicates were performed, the order of application was randomised to avoid habituation.

For both conditions 300 mL of CO_2 were used as carbon source.

***Table 7.1**. Program of experiments*

Condition name	Original reactor	Actual pH	
		pH	SD
7.5	P7	7.2	0.27
6.5	P5	6.49	0.10
5.5		5.64	0.05
4.5		4.48	0.08
3.5		3.46	0.05

7.2.3. Analytical techniques

Liquid samples were collected at the end of each batch cycle to analyse total organic carbon (TOC), total inorganic carbon (IC), total nitrogen and volatile fatty acids (VFAs) from C_2 to C_6. Also dissolved oxygen, redox, pH and ammonium were measured following standard methodologies [21].

At the end of each cycle, the gas bag was emptied and the amount of gas (V_g) was measured and gas composition determined by a gas chromatography [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 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 ºC.

7.2.4. Molecular biology techniques

Cathode samples were taken from both reactors. Samples of reactor P7 were taken at the end of the experiment (cycle 51) while samples of reactor P5 were taken at the end of the conditions 5.5 and 3.5 (Table 7.1).

These samples were used to characterise the microorganisms that had developed at the methane-producing biocathode. After extractions microbial communities were analysed by high-throughput sequencing of 16S rRNA gene-based massive libraries. 16 rRNA universal primers were V4 515F-806R for bacteria and V4 349F-806R for archaea. Quantification of total bacteria and archaea was performed by qPCR.

7.3. Results and discussion

7.3.1. Impact of pH on the start-up process

After inoculation, both reactors were operated in batch conditions for 14 cycles (Figure 7.2). Results seem to indicate that the acidic conditions in P5 favoured both current density and methane production. By the end of start-up period, P5 produced 38% more methane and 35 % more current density per cycle than P7. In addition, CH_4 production started earlier in P5 (cycle 3), while in P7 in took 4 additional cycles. The average of the efficiencies for each reactor (once

they started producing methane) revealed that a more acidic condition contributed to higher process efficiency: 63% in P5 and 49% in P7.

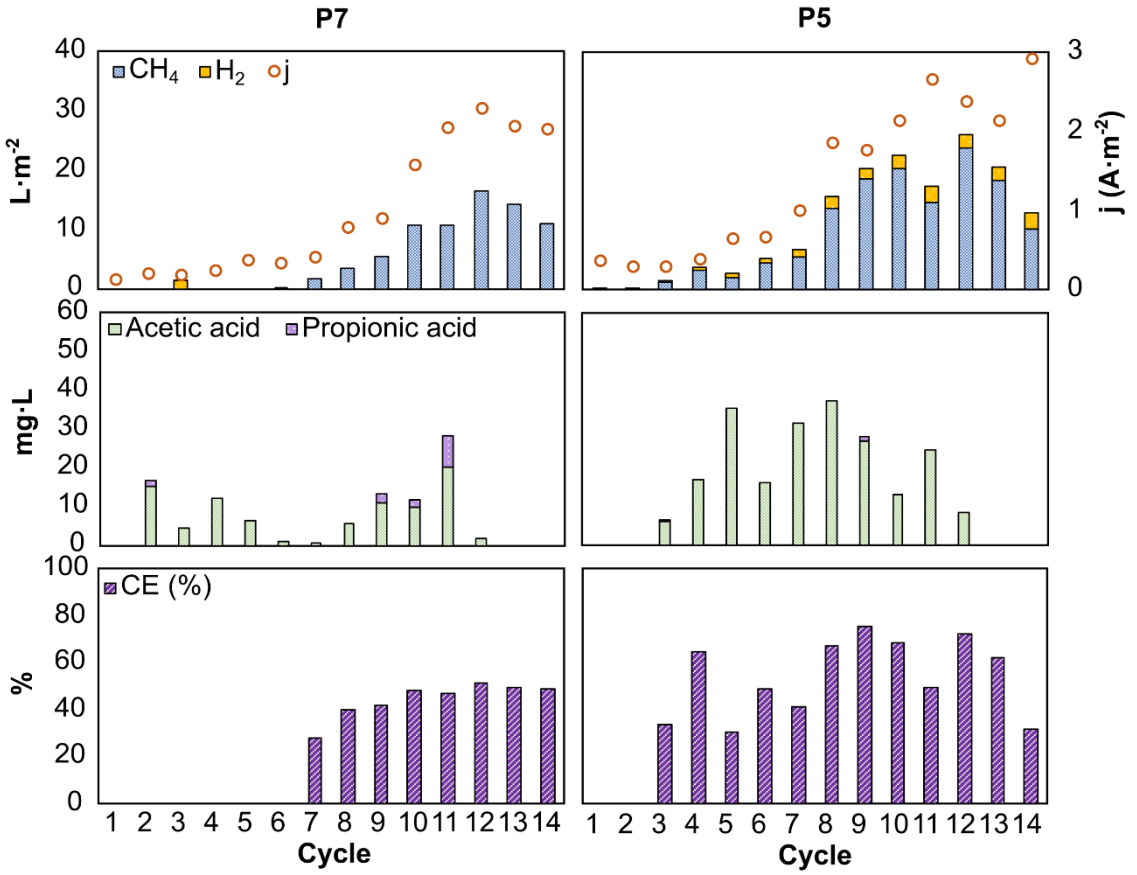

Figure 7.2. Top: *Methane and hydrogen production per electrode surface area and current density.* ***Middle:*** *acetic and propionic acid production.* ***Bottom:*** *and current efficiencies (CE).*

The acidic environment in P5 also seemed to stimulate the production of VFAs. Cumulative acetic acid production along the 14 cycles was 216.8 $mg \cdot L^{-1}$ in P5, well above the 88.3 $mg \cdot L^{-1}$ measured in P7. However, P7 produced a significant amount of propionic acid 13.5 $mg \cdot L^{-1}$, a VFA that was barely detected in P5.

In any case, from cycle 11 onwards, the production of VFAs in P5 decreased as methane production reached stabilisation, and it totally stopped on the last two cycles (13 and 14). A similar behaviour has been reported in previous studies: in [22] for instance, the increase in methane content during the start-up coincided with the decline in acetic acid levels indicating a possible displacement by competitive advantage of methanogenic organisms over acetogenic organisms. Several authors have proposed that methane and VFAs production in MES/EM systems are mediated by hydrogen [23–25], so most probably both groups of microorganisms would be competing for this intermediate in our reactors. It is known that acetogenic bacteria have a growth rate almost three times higher than methanogenic archaea [26]. In addition, it seems that acetogens have a kinetic advantage over methanogens when the partial pressure of H_2 is high [22,27], all of which would explain the production of VFAs during the start-up. Despite these competitive advantages of acetogens, methanogens can gain more energy than acetogens from the consumption of H_2 and CO_2 [22]. In the long run, we can expect a gradual increase in methane production accompanied by a gradual drop in VFAs production, which is consistent with the results presented in Figure 7.2.

7.3.2. Impact of acidic pH on EM

Current density began to be repeatable in both reactors through cycles 10 to 14, so it was assumed that the cathodic biofilms were mature enough to start the next experimental phase. In this phase, both reactors were operated in batch mode too (5 days duration), and the pH of the catholyte in reactor P5 was

gradually modified (pH in P7 remained around 7) as shown in Table 7.1. This resulted in significant changes in its performance (Figure 7.3**)**. On the one hand, current density tended to increase as the pH drops, peaking at pH 4.5. This can be possibly explained by the greater availability of H^+ ions at low pH, which reduces concentration overpotentials and favours current production. However, when the pH was further reduced to 3.5, current declined sharply, which might be indicating unfavourable physiological conditions for electrotrophic bacteria that limit their catalytic activity. Interestingly, methane production followed a similar trend, and at pH 4.5 the production of this gas doubled that at pH 7.5. This result corroborates the syntrophic relationship between the hydrogenic electrotrohs and the hydrogenotrophic methanogens [28,29], that would be probably using hydrogen as an intermediary metabolite.

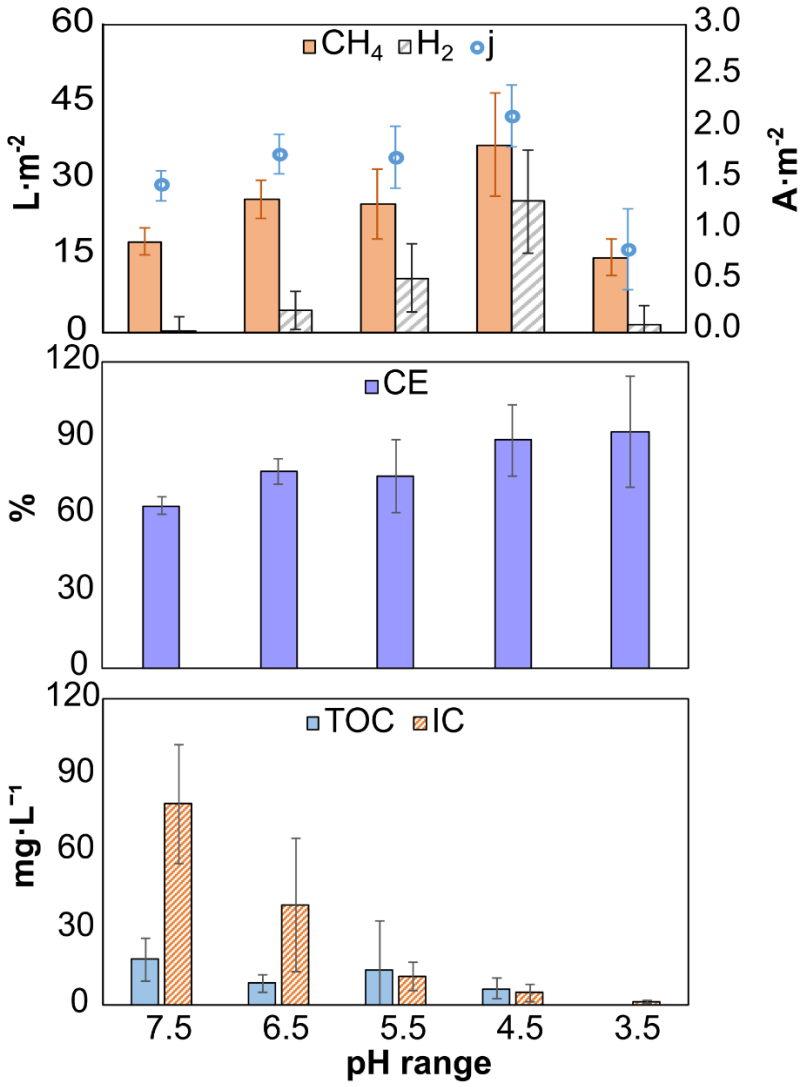

Figure 7.3. Top: *Averaged methane and hydrogen production per electrode surface area ($L \cdot m^{-2}$) and current density for each experimental condition.* ***Middle:*** *current efficiency.* ***Bottom****: total organic carbon (TOC) and inorganic carbon (IC).*

Cyclic voltammetry analyses would confirm this hypothesis, as the resulting cyclic voltammograms (CV) shown in Figure 7.4 exhibited the characteristic reduction waves associated to the H_2 evolution reaction [30,31]. In addition, the CVs at pH 6.5 and 5.5 CV showed the largest current peaks, being both notably larger than the peak obtained at pH 7.5, which is coherent with the

current densities obtained during the batch tests (Figure 7.3). Moreover, the low signal at pH 3.5 would also be consistent with the inhibition of electrotrophs as discussed above.

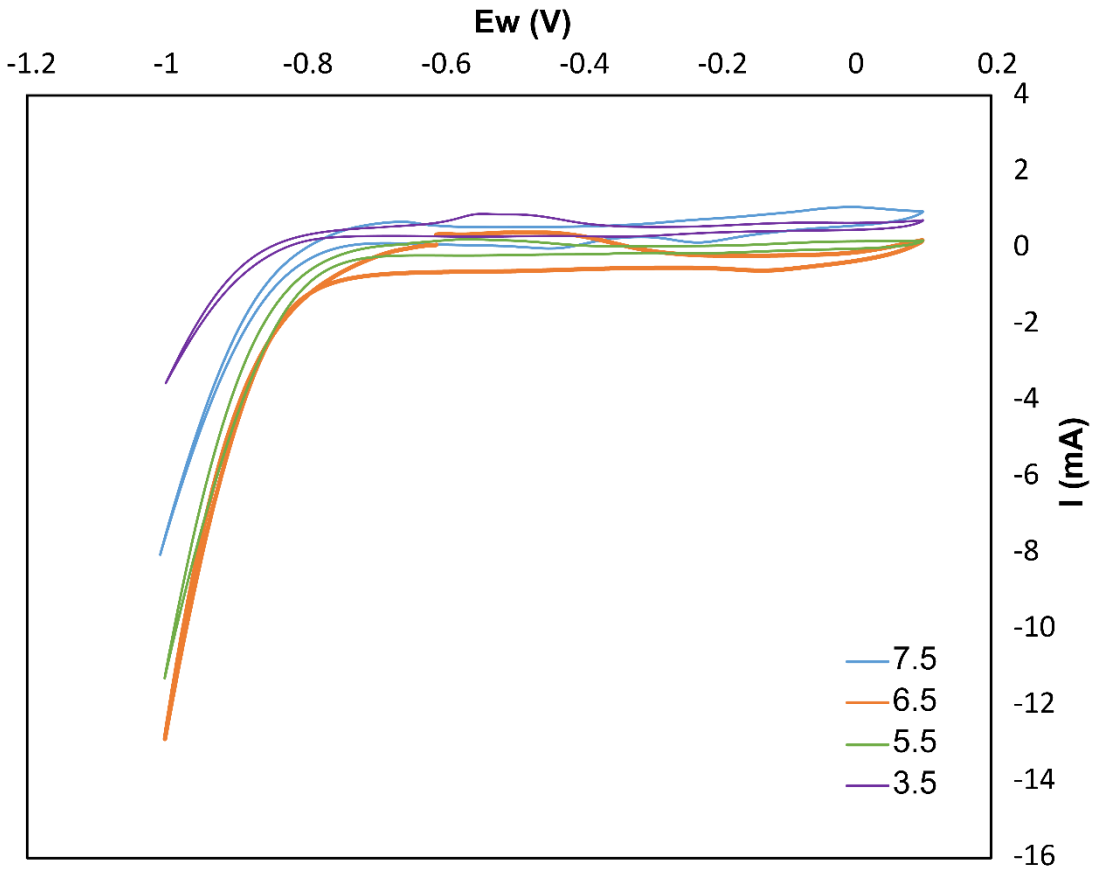

Figure 7.4. *Current voltammetry for each experimental condition.*

It is worth noting that at pH 3.5 both communities are well below their optimal pH range (4.0 to 7.0 for electrotrophs [32] and 6.5 to 8.0 for methanogens [13]), so the fact that at pH 3.5 we still measure some current and methane can only be attributed to the relatively higher local pH on the vicinity of the cathode (where the biofilm develops compared to the bulk), that would provide a favourable environment for the proliferation of both groups. Previous studies with

microsensors showed that in hydrogenotrophic methanogenic reactors the pH increases in the biofilm with respect to bulk [27] which would support this hypothesis.

It is also interesting to note that the amount of H_2 in the headspace experienced an increase as the pH drops (Figure 7.3 top), suggesting that methanogens could not absorb the excess of hydrogen production at low pHs. This is probably because of a relatively low growth rate of archaea (methanogens) compared to bacteria (electrotrophs) [26]. The efficiency of the process increased as the experimental condition became more acidic, being maximal for pH 3.5 (Figure 7.3 middle), which implies that even though hydrogen and methane production decrease sharply at this pH, the electrons are being used more efficiently in the production of these gases.

IC and TOC also decreased as the catholyte became more acidic (Figure 7.3 bottom). At pH 7.5, TOC reached its maximum value (18 $mg \cdot L^{-1}$) as the production of some VFAs was detected (but only in a few cycles and sporadically): mainly acetic acid (7.1 $mg \cdot L^{-1}$), butyric acid (2.6 $mg \cdot L^{-1}$) and propionic acid (1.1 $mg \cdot L^{-1}$) (Figure S7.1 in Appendix 3). The decrease in TOC can be attributed to several factors. On the one hand, the low concentration of IC at low pH [33] would mean a lower carbon availability for the synthesis of organics. On the other hand, it is known that low pH inhibits the homoacetogenic microorganisms that may favour the proliferation of hydrogenotrophic methanogens. Indeed, a shift from acetoclastic to H_2-dependent methanogenesis have been described when pH decreases at a range of 4.7 and 3.8 [34].

7.3.3. Impact of acidic pH on EM microbiology

Relative abundances analyses of the main genera present in the cathodic biofilms revealed large differences in comparison to the microbial communities originally present in the inoculum (Figure 7.6). The most striking feature is perhaps the large proliferation of *Methanobacterium* on the biofilms cultured at low pH (5.5 and 3.5). *Methanobacterium* is a hydrogenotrophic methanogenic archaeon [28,35–37] whose presence corroborates the hypothesis that H_2 acts as an intermediate in the transfer of electrons, as it was previously discussed.

It has been reported that 7 is the optimal pH for the proliferation of this genus, being inhibited at pH 5.5 or below [38–40]. The higher local pH on the surface of the cathode (compared to the bulk) as noted above, in combination with the easy availability of H_2 on the biofilm, may have favoured its abundance against other methanogenic archaea such as *Methanobrevibacter* or *Methanosaeta* [41,42] and could explain why pH 5.5 favoured *Methanobacterium* and why more acidic environments (condition 3.5) caused a decrease.

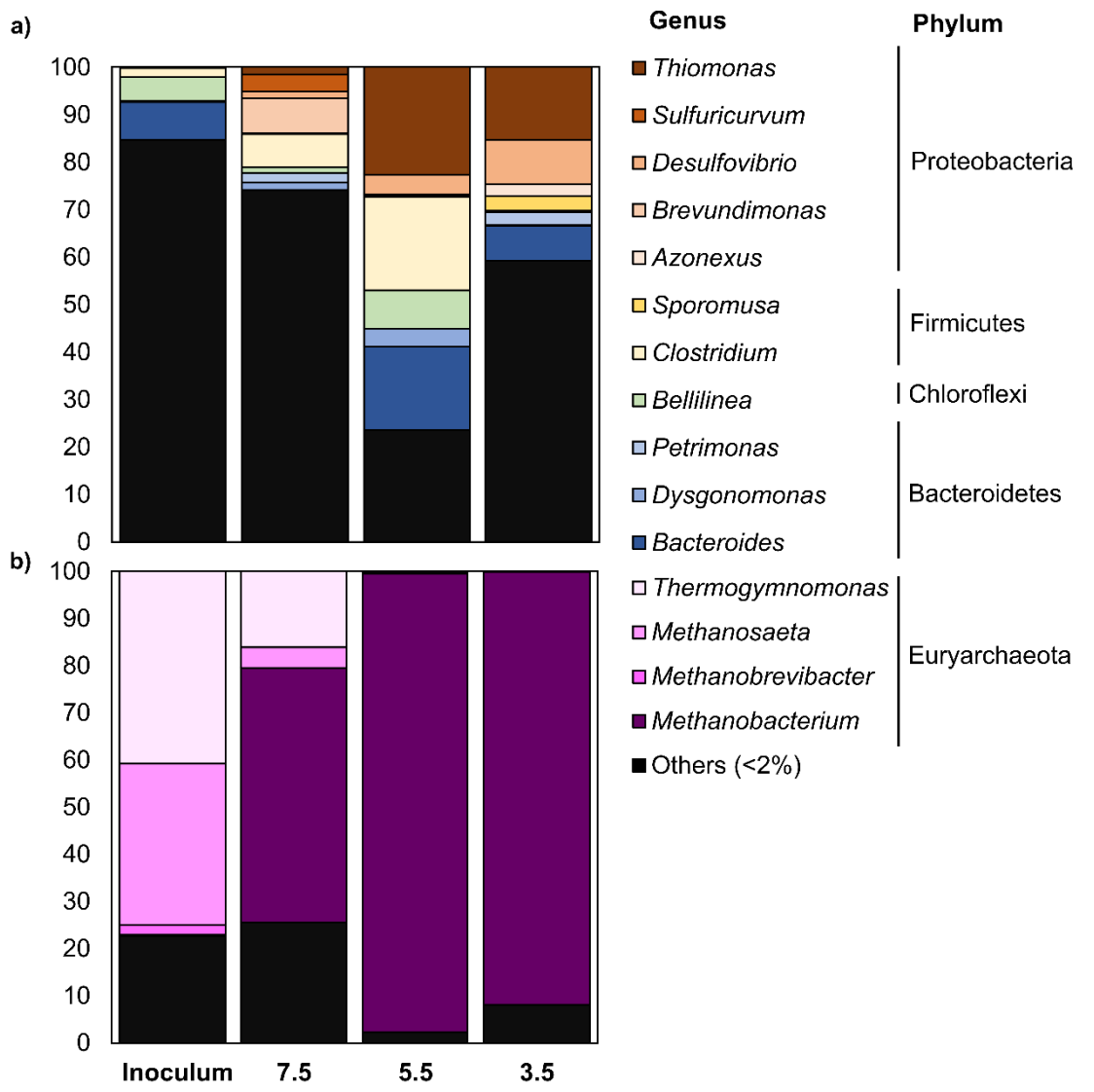

Figure 7.5. *a) Relative abundance for bacteria; and b) relative abundance for Archaea.*

Regarding bacteria, it is noteworthy the relatively high abundances of *Desulfovibrio* and *Petrimonas at pH 5.5 and 3.5.* These are two electroactive microorganisms that have been described before in biocathodes as being capable of producing H_2 via direct extracellular electron transfer [31,43,44], and whose presence in a significant proportion in our biocathode supports the theory

of a syntrophic relationship with *Methanobacterium*. *Desulfovibrio* species have been found to be capable of growing in biocathodes at a pH as low as 3.0 [45], although most of its members have an optimal growth range between 5.5 and 7 [46]. For *Petrimonas spp*., the optimal pH is found in the range between 7.1 and 7.8 [47]. As discussed before, the relatively high pH on the surface of the cathode (compared to the bulk electrolyte) would provide a suitable environment for the proliferation of these two genera when the bulk pH drops to 5.5 and 3.5 (Figure 7.5). However, quantitative analyses (Figure 7.6) revealed a notable decline in the total bacterial population (of almost two orders of magnitude), which is not entirely coherent with the CV results (Figure 7.4.) and with relatively high current densities measured in the batch tests at pH 5.5 and 3.5. It can be hypothesized that *Petrimonas* and *Desulfovibrio* mainly grow on the deep layers of the cathodic biofilm in close contact with the cathode, where they can perform the direct extracellular electron transfer and where they are protected by the more superficial layers that are more exposed to the bulk pH.

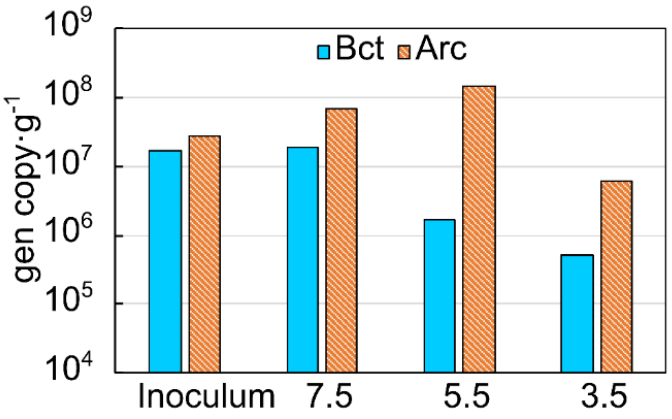

Figure 7.6. *qPCR in terms of total bacteria (Bct) and archaea (Arc) for inoculum and each experimental condition.*

Clostridium, a genera capable of reducing CO_2 to acetate in the presence of hydrogen [1,21], showed also a significant abundance, especially at pH 5.5 and could be responsible for the presence of the acetate detected in some experiments (Figure S7.1 in Appendix 3) ,and that could be a potential substrate for acetoclastic methanogens. However, the virtual disappearance of the *Methanosaeta* (a well-known acetoclastic methanogen [48]) initially present in the inoculum rules-out this possibility.

Finally, the biocathodes also provided a suitable environment for a notable proliferation of *Thiomonas spp.,* an extremophile genus found in biocathodes and related to sulphide removal processes [49] whose role in our biocathodes is not clear.

7.4. Conclusions

Two MES/EM reactor were inoculated at a pH 5.5 and 7.5 respectively. pH did not seem to have any impact on product selectivity: in both situations, and during the first 11 cycles of batch operation the main end products were acetate and methane. Nevertheless, the more acidic conditions contributed to a faster start-up and a faster product formation. In addition, acetate was almost totally displaced by methane after 11 batch cycles. In subsequent tests, as the pH of the catholyte was gradually reduced, current density and methane production increased and peaked at a pH of 4.5. Further reducing pH to 3.5 resulted in a notable deterioration of performance, although the cathodic efficiency improved slightly, which reveals that despite the decline in production rates, the current is used efficiently in the production of methane.

The acidic conditions also favoured the proliferation of the hydrogenotrophic methanogenic archaea *Methanobacterium*, growing, probably, in syntrophy with hydrogen producing bacteria such as *Desulfovibrio* or *Petrimonas* confirming thus the role of H_2 as the main intermediate between electron uptake and CO_2 reduction.

7.5 References

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CHAPTER 8

ELECTROMETHANOGENESIS FOR THE CONVERSION OF HYDROTHERMAL CARBONIZATION EXHAUST GASES

8.1. Introduction

Depletion of fossil fuels and the contribution of carbon dioxide emissions to climate change are stimulating the transition from traditional petrochemical refineries to biorefineries [1]. These facilities can contribute to meet the ambitious goals set by the European Union on the reduction greenhouse gases emissions and the implementation of a circular economy, especially when using wastes (instead of crops) as feedstock [2,3]. The list of industrial processes that produce CO_2-rich waste streams is certainly large, and the ability of some living microorganisms to assimilate CO_2 opens the way for using these wastes as chemical raw materials [4].

Within this context, hydrothermal carbonization (HTC) represents a very attractive process [5]. HTC is a thermochemical technology that offers a sustainable and cost-effective solution for waste management while pursuing the concept of a carbon-neutral society [6]. This process, that occurs under autogenous pressure and at moderate temperatures (150 °C - 300 °C) compared to conventional pyrolysis (400 °C - 600 °C), can convert organic wastes into three different products [7,8]. The main product is the solid phase, commonly known as hydrochar, which can find applications as solid biofuel, low-cost adsorbents or soil amendment among others [9]. There is also a liquid phase that usually contains a wide spectrum of valuable chemicals for biorefineries [10,11], and a gaseous phase that is composed mainly of CO_2 (ca. 85-95%) with minor proportions of other gases such as CO, CH_4 or H_2 [8] and small traces of hydrocarbons [12]. Because of its large CO_2 content, the gaseous phase is commonly seen as a waste. However, as previously demonstrated [13], the valorisation of this side product could improve the overall economy of HTC, while

avoiding CO_2 emissions to the atmosphere. Despite that, only a few works have explored the possibilities of HTC off-gas conversion and valorisation [14,15]. One example is the work of González-Castaño and colleagues, who showed how the Reverse Water Gas Shift reaction pathway can be implemented after the HTC process to obtain syngas [15].

HTC off-gas also represents an ideal feedstock for methane production through electromethanogenesis (EM) [16,17]. EM is a biologically mediated process that results in the conversion of CO_2 to methane on the cathode side of a bioelectrochemcial systems (BES). For more details on BES and EM the reader is referred to [18]. EM has aroused significant interest among scientists and engineers because of its environmental and economic potential. It can proceed at room temperatures and pressures and involves bacteria as catalysts, all of which suggest that EM can become a more cost-effective and environmentally friendly method of methane production compared to conventional technologies [17]. Despite that, technical and economic limitations still remain, and the scaling up of this technology represents a major challenge [19].

Previous studies have shown that methanogenic biocathodes can be successfully fed with synthetic mixtures of CO_2 and other gases [20–22], and it has been even demonstrated that the CO_2 present in a real biogas is a suitable substrate for EM [23,24]. However, the use of real CO_2-rich waste streams (such as the HTC off-gas) as feedstock has not been yet explored. This chapter explores the technical feasibility of using real HTC off-gas as a raw material for EM, paying special attention to the impact that this gas has on process performance and on the cathodophilic microbial communities.

8.2. Materials and methods

8.2.1. Reactors design

The experiments were conducted using two standard H-type reactors (referred to as R1 and R2 throughout the paper) with an internal volume of 500 mL per chamber. The biocathodes (working electrodes) used in these experiments were inoculated with the biofilm scratched from the biocathodes used in a previous experiment [20]. Each of the electrodes consisted of two pieces (2 x 8 cm) of carbon felt (SGL Group, Germany) attached by titanium wire and suspended inside the cathodic chamber with a graphite rod. Prior to inoculation, the electrodes were pretreated by subsequent immersion in nitric acid 1 M, acetone 1 M and ethanol 1 M during 24 h each to avoid hydrophobicity and impurities [25]. The counter electrodes (CE) were made of a 2 x 2 cm platinum mesh (Goodfellow, UK) suspended inside the anodic chamber with titanium wire. A pretreated cation exchange membrane (CMI7000, Membranes International, USA) was used to separate the anodic and cathodic compartments (Figure 8.1).

Both reactors were operated on a three-electrode configuration using a Biologic VSP potentiostat (Biologic, France) and EC-Lab® software (ver. 11.31). An Ag/AgCl commercial reference electrode (Sigma-Aldrich, USA) (0.20 vs. SHE; the stability of the reference electrode was checked prior to every batch cycle) was used as reference electrode.

Appropriate connections and sealing were designed for sampling ports and substrate supply as illustrated in Figure 8.1. Gas was collected using a 1 L gas bag (Ritter, Germany). Reactors were placed inside a phytotron (Fitotron, Sanyo, Osaka, Japan) that maintained temperature constant at 30±1 ºC. The

catholyte was continuously stirred (200 rpm) using a magnetic stirrer (RO15, IKA. Staufen, Germany) in order to prevent mass transfer limitations on current efficiency [26].

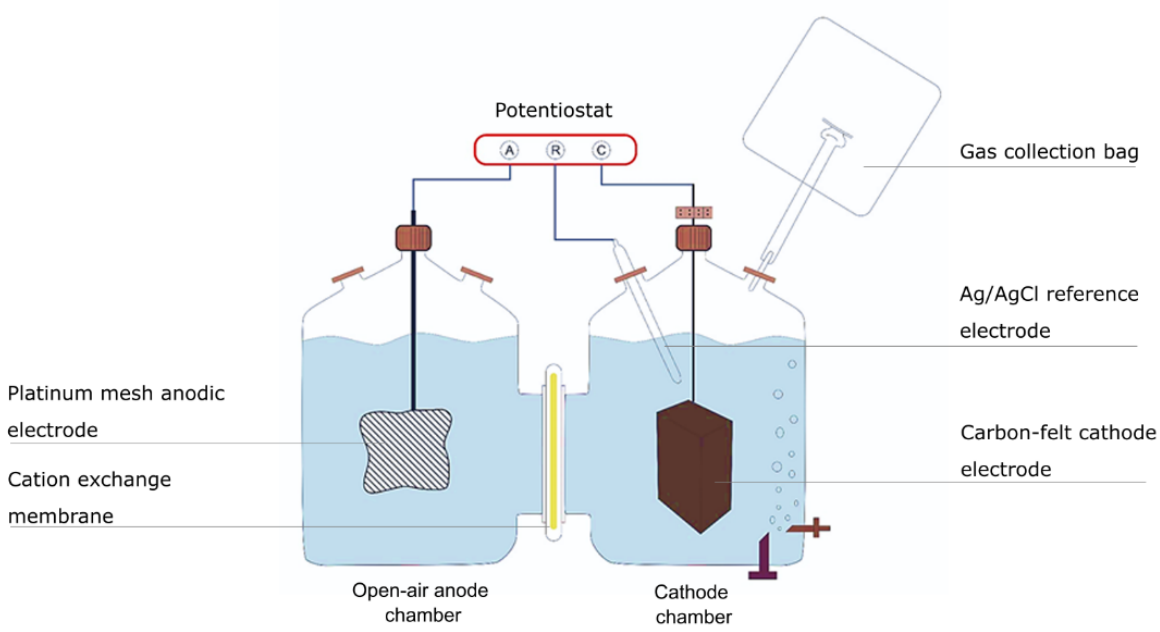

Figure 8.1. *Reactor diagram.*

8.2.2. HTC off-gas

The feedstock used for the production HTC off-gas consisted of the pruning of arboreal biomass collected form a nearby poplar farm. 50 g of biomass were mixed with 1000 mL of deionised water at a 1/20 biomass/water ratio in a 2 L stirred pressure reactor (APP Parr reactor, Parr instrument company, Moline, IL, USA) operated at 250°C during 1 hour (the reaction parameters based on previous experiences [27]). The off-gas was collected in a 1 L Tedlar gas bag,

and consisted of a mixture of: CO_2 90.10% ± 1.72; CO 9.19% ± 1.68; H_2 0.14 ± 0.04; CH_4 0.13% ± 0.01; and traces of N_2.

8.2.3. Operation

Reactors were operated in batch-mode in cycles of 7 days. During the start-up, reactors were fed with 300 mL of CO_2 gas as sole carbon source and were allowed a stabilization period of 15 batch cycles, after which current density profiles were fairly repeatable between cycles and the experimental phase itself began (see SI Fig S1).

During the experimental period reactors were batch-fed with 300 mL of a mixture of HTC offgas and pure CO_2. The HTC offgas proportion was progressively increased until the feed was exclusively HTC offgas (Tests 1 to 5 in Table 8.1). In the final test (Test 6), pure CO_2 was fed again to evaluate the eventual reversibility of the process as well as to infer possible toxic effects from the HTC.

Table 8.1. *Proportions of pure CO_2 and HTC in the fed-gas during the experimental phase.*

	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
% CO_2	100	80	50	25	0	100
% HTC	0	20	50	75	100	0

8.2.4. Analytical techniques

Liquid samples were taken at the end of each cycle to analyse total organic carbon (TOC), total inorganic carbon (IC), total nitrogen (TN) and volatile fatty acids (VFAs) from C_2 to C_6. Dissolved oxygen, redox, pH and were measured following standard methodologies [25].

At the end of each batch cycle, the amount of gas in the bag (V_g) was measured and gas composition was determined by means of a gas chromatography [25]. The volume of hydrogen and methane produced in each cycle was calculated from V_g 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 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} and a temperature of 30 ºC.

8.2.5. Molecular biology techniques

At the end of test 6 (Table 8.1), 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 and followed at the end of the experimental period by high-throughput sequencing of 16S rRNA gene-based massive libraries. 16 rRNA universal primers were V4 515F-806R for bacteria

and V4 349F-806R for archaea. Quantification of bacteria and archaea was performed by qPCR.

8.3. Results and discussion

Before the experimental phase began, the biocathodes were allowed a stabilization period of 15 batch cycles (7-day duration of each cycle) during which the reactors were fed with pure CO_2 as the sole carbon source. By the end of this period, the current density profiles tended to be repeatable between cycles (see Figure S8.1 in Appendix 4), indicating that the biocathodes were mature enough to initiate the experiments.

8.3.1. The impact of gradually increasing the amount of HTC off-gas in the feeding

During the experimental phase, the reactors were batch-fed (7-day duration of each cycle) with 300 mL of a gas mixture consisting of CO_2 and increasing amounts of HTC gas (Table 1). Figure 8.2a shows how current density declines steadily with the HTC, falling from about 1.5 A m^{-2} when no HTC is present to 0.6 A m^{-2} when only HTC is fed to the reactors, which reveals a negative impact on the microorganisms that are directly involved in current production (see Figure S8.2 in Appendix 4). Methane production was also affected by the presence of HTC in the fed-gas (Figure 8.2b), decaying form about 2.3 mmol per cycle (0% HTC) to 1.2 mmol per cycle (100% HTC). This decline in both, current density and methane production, might be connected to

the presence of carbon monoxide (CO) in the HTC (Figure 8.3), as CO can inhibit the activity of metal-containing hydrogenases that catalyse the reversible conversion of protons and electrons to hydrogen [28,29]. Nevertheless, when pure CO_2 was fed again to the reactors at the end of the experimental phase, current density and methane production returned to values similar to those observed before HTC gas was fed, which suggests that the changes induced by HTC were reversible.

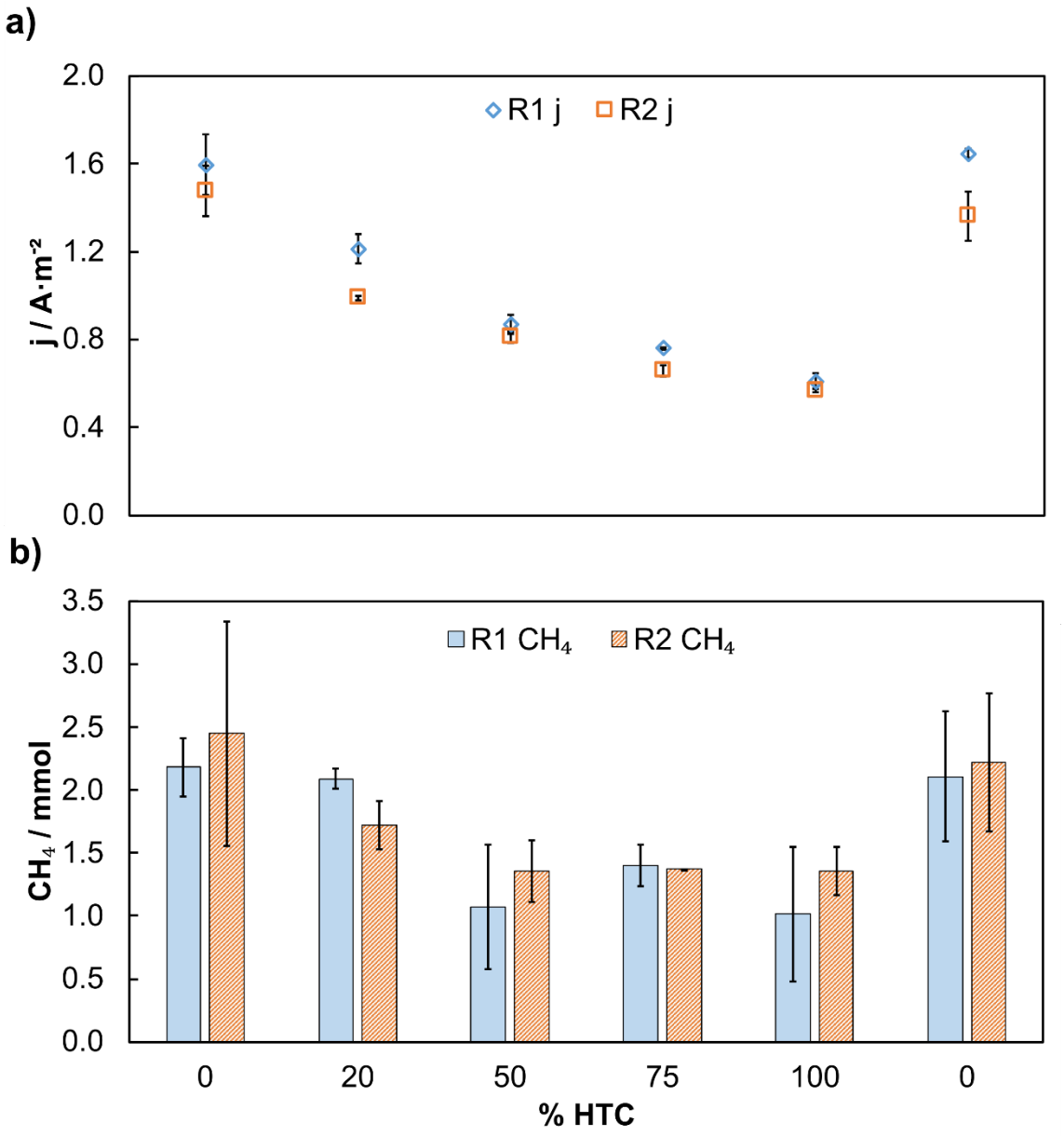

Figure 8.2. *Average current density (top) and methane production (bottom) as a function of the proportion of HTC feed to the reactors. The last condition represents the return to a feeding with pure* CO_2

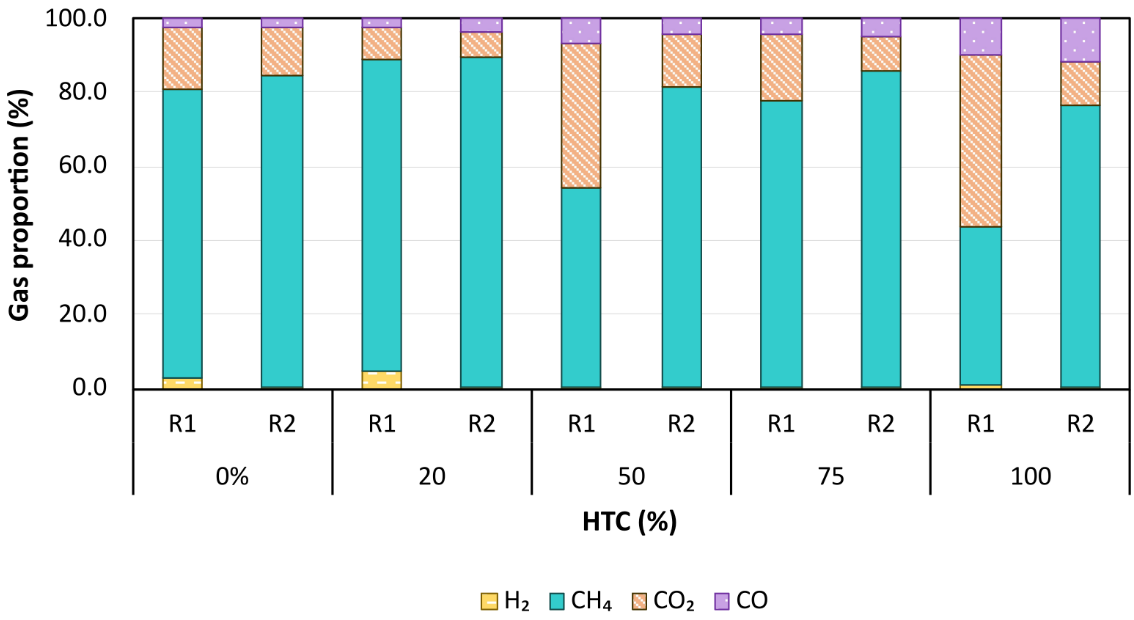

***Figure 8.3**. Gas proportion as a function of the amount of HTC off-gas fed to the reactors.*

It is interesting to note that, in contrast to current density and methane production, the coulombic efficiency improved with HTC (Figure 8.4), which apparently means that HTC promotes a more efficient use of current, probably as a result of less electrons are being diverted to biomass.

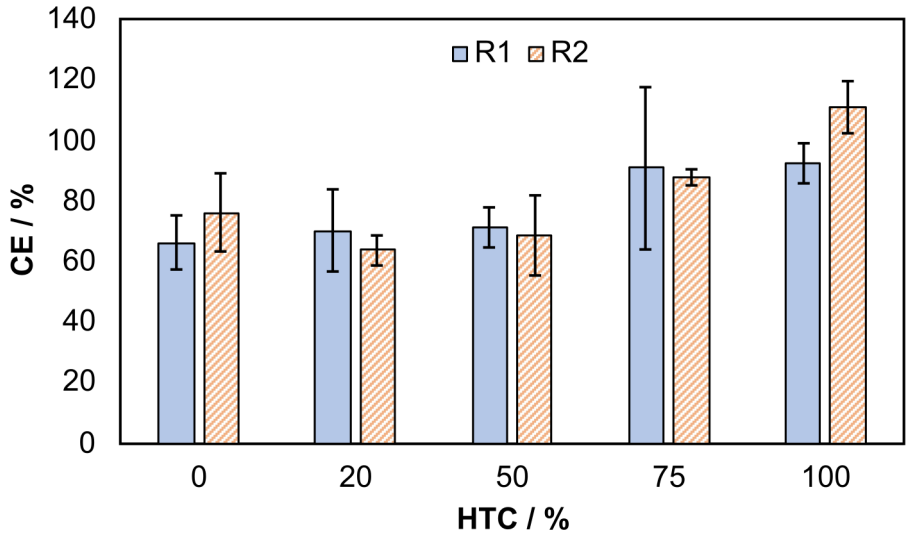

***Figure 8.4**. Average values of coulombic efficiency (CE) as a function of the proportion of HTC off-gas fed to each reactor*

This hypothesis is coherent with the loss of biomass detected by qPCR analyses as it will be discussed below. However, it does not explain why HTC concentrations above 75% resulted in coulombic efficiencies greater than 100% (Figure 8.4). Under these circumstances, a more plausible explanation can be traced to the potential role of CO as an electron donor (alternative to the cathode) that "artificially" increases the CE. Indeed, previous studies on CO fermentation have found that carboxydotrophic bacteria can use CO to produce acetate, H_2, and CH_4 [30,31]. To make sure whether this might be happening in our reactors, we operated them for two cycles with 300 mL of HTC and in the absence of any applied voltage. This resulted in the production of significant amounts of CH_4 that can only be attributed to CO fermentation (Figure 8.5). Moreover, the amount of methane measured in these tests is stoichiometrically coherent with the amount of CO in the HTC.

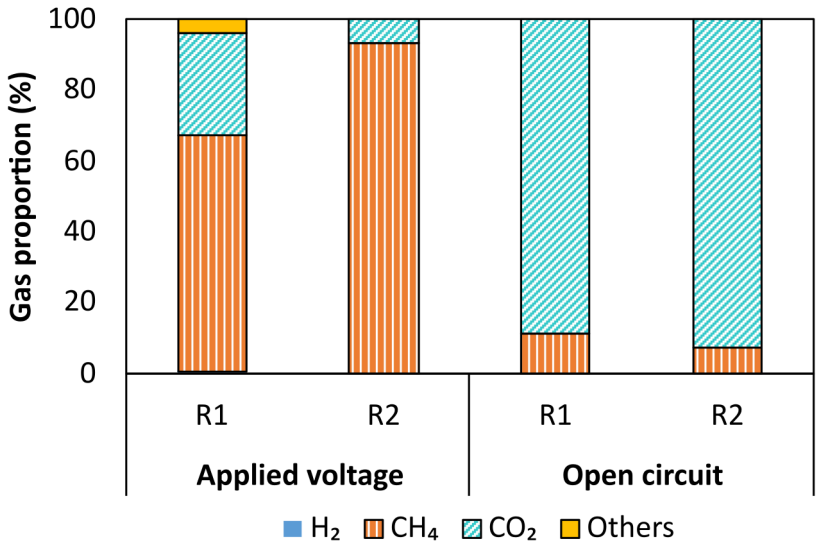

Figure 8.5. *Gas composition under applied voltage and open circuit conditions using 100% HTC off-gas as substrate.*

CO conversion to methane in the presence of hydrogen (according to Equation 8.1 [35]) can also explain the increase in the CE. This route requires only 6 moles of electrons per mole of methane ─instead of the 8 moles of electrons for CO_2 route─, which represents a 25% reduction in current usage, and therefore an increase in the CE.

$$CO + 3H_2 \leftrightarrow CH_4 + H_2O \qquad \text{Equation 8.1}$$

As the catholyte is replace by a fresh nutrient solution at the beginning of each cycle, the accumulation of reducing power between cycles via extracellular matrix or through the loss of biomass can be ruled out as significant source of reducing power for methane production.

8.3.2. CV and microbiology analyses

CV and microbiology analyses were performed to deepen the understanding of the impact of the HTC gas on the bioelectrochemistry of the methanogenic biocathode. Figure 8.6 represents the voltammograms recorded at the beginning of each batch cycle for the different HTC fractions. Both reactors showed a large reduction peak at potentials below −0.9 V that has been usually associated to H_2 evolution [32]. As our reactors were operated at −1.0V, this peak confirms that methanogenesis is occurring through the H_2-mediated indirect electron transfer (IET) mechanism [33]. In addition, the size of the hydrogen peak decreased with the fraction of HTC in the fed-gas, which is consistent with the results of the chronoamperometries presented in Figure 8.2. The impact of HTC was more apparent on R2 (37% decrease in peak current) that on R1(27% decrease), so it seems that R1 might have developed a more robust and resilient electrotrophic biofilm. However, the microbiology analysis (Figures 8.7 and 8.8) did not provide a clear support to this hypothesis.

CVs showed another reduction peak ─much smaller than that associated to hydrogen evolution─ at about −0.6 V. This peak could most probably be attributed to electromethanogenesis via the direct electron transfer (DET) mechanisms [32], although its contribution to electromethanogenesis would be marginal compared to the IET mechanism [20,33]. In addition, the size of the DET peak in R1 varied with the amount of HTC, although no apparent trend was visible. An oxidation peak only appeared in R1, with no apparent trend either. These two peaks, that seem to be inter-related, disappeared almost completely when HTC proportion was 100%, which might be indicating an adaptation process.

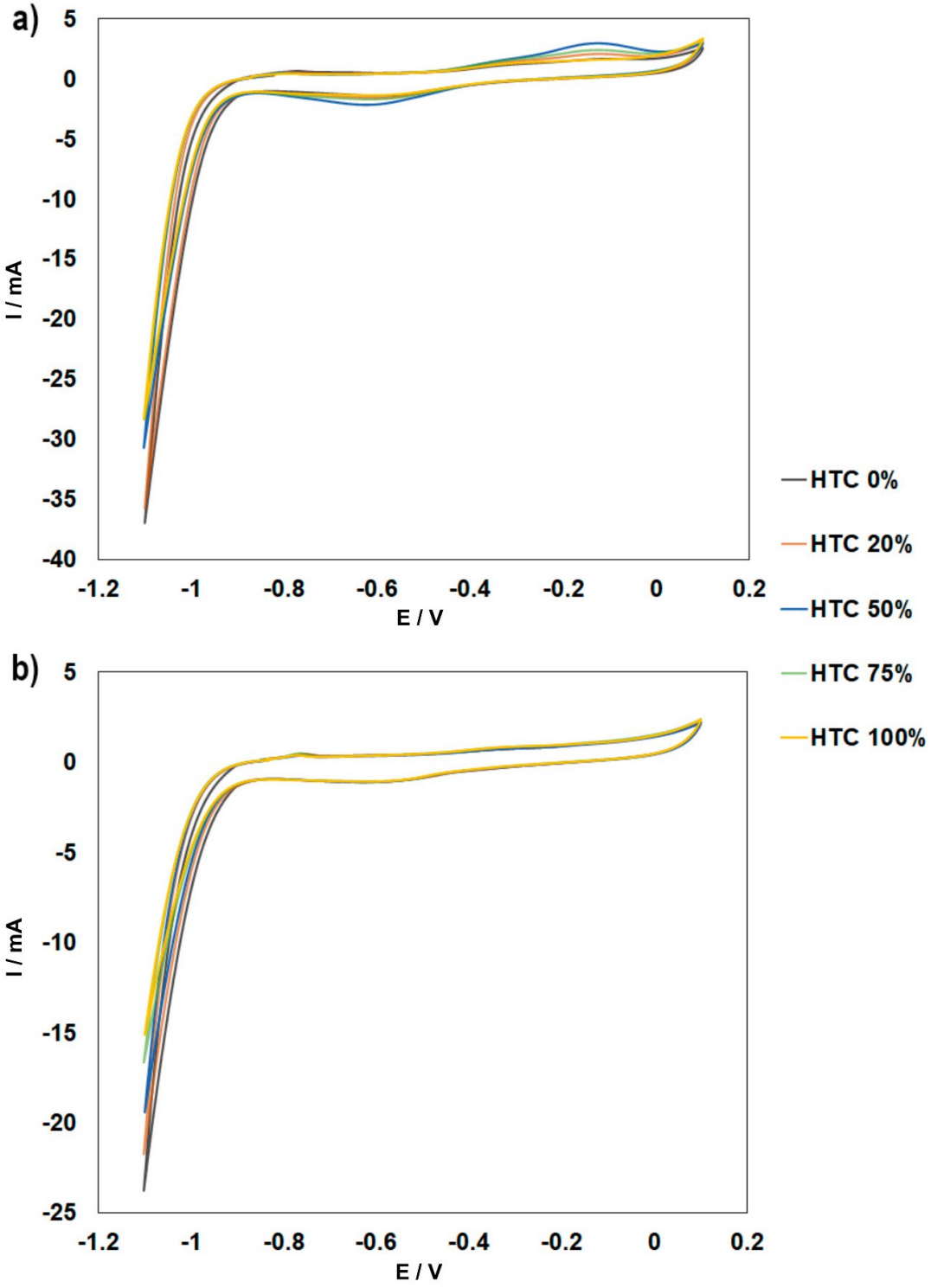

***Figure 8.6**. CVs for R1 (top) and R2 (bottom) at the different HTC proportions in the fed-gas.*

qPCR analysis revealed that both, Bacteria and Archaea were seriously damaged by the presence of HTC off-gas in the fed (Figure 8.7). However, the impact ─measured in terms of the decrease in the number of gene copies─ was unequal for both groups; while for bacteria the introduction of HTC meant a loss of 85% and 66% (R1 and R2, respectively) of their communities, for archaea it meant 96% and 97% (R1 and R2, respectively). This loss in biomass would explain the poor performance, but also ─at least partially─ the better CE values observed with HTC off-gas as discussed above. Previous studies have pointed out the ability of CO to inhibit methanogenic organisms, which could explain the greater decrease in archaea [34].

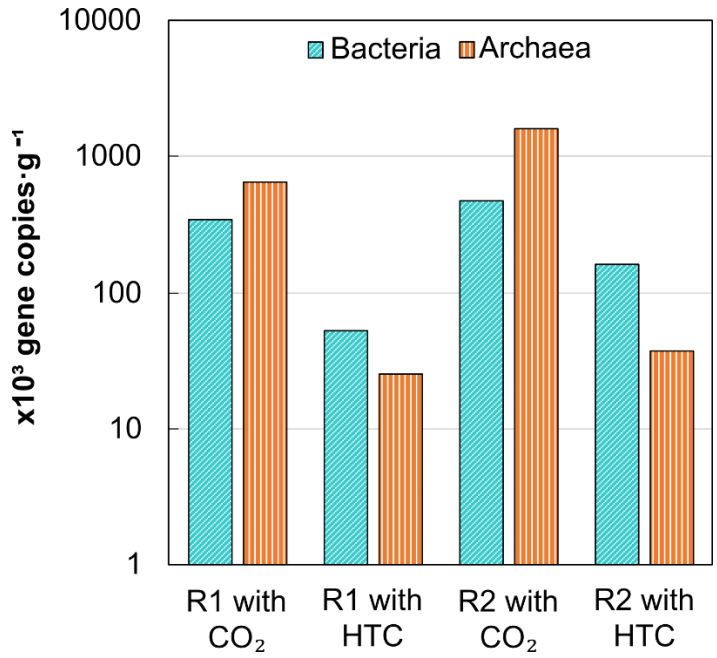

Figure 8.7. *Gene copy number in CO_2 and HTC fed reactors obtained by qPCR analyses*

Relative abundance analyses (Figure 8.8) indicated that *Methanobacteriaceae* dominated archaea in both reactors, although its proportion experienced a notable decay with HTC off-gas: from 56.6% to 44.6% in R1 and from 56.8% to 30.1% in R2. Nevertheless, its relatively large presence is consistent with the hypothesis that H_2 acts as an intermediary in the electron transfer [32,35,36], as most members of this family are hydrogenotrophic methanogens. Moreover, some species of this family have been reported to grow on CO as the sole carbon source while producing methane, although they appear to be not very metabolically efficient [29].

The *Methanosacetaceae* family, all of its members use acetate as their sole source of energy [37], completely disappeared after HTC off-gas was fed, which can be related to the total absence of *Clostridiaceae*. (Fig. 7) Indeed, many species within the later are well known acetogenic bacteria [38], so there might be a syntrophic link between this two families that was broken with the presence of HTC, causing them both to disappear. This result contrasts with [39], where the authors proved that the electroactive bacteria of a CO-fed microbial electrosynthesis biocathode not only tolerated CO, but they were able to convert it into acetate (and other volatile fatty acids).

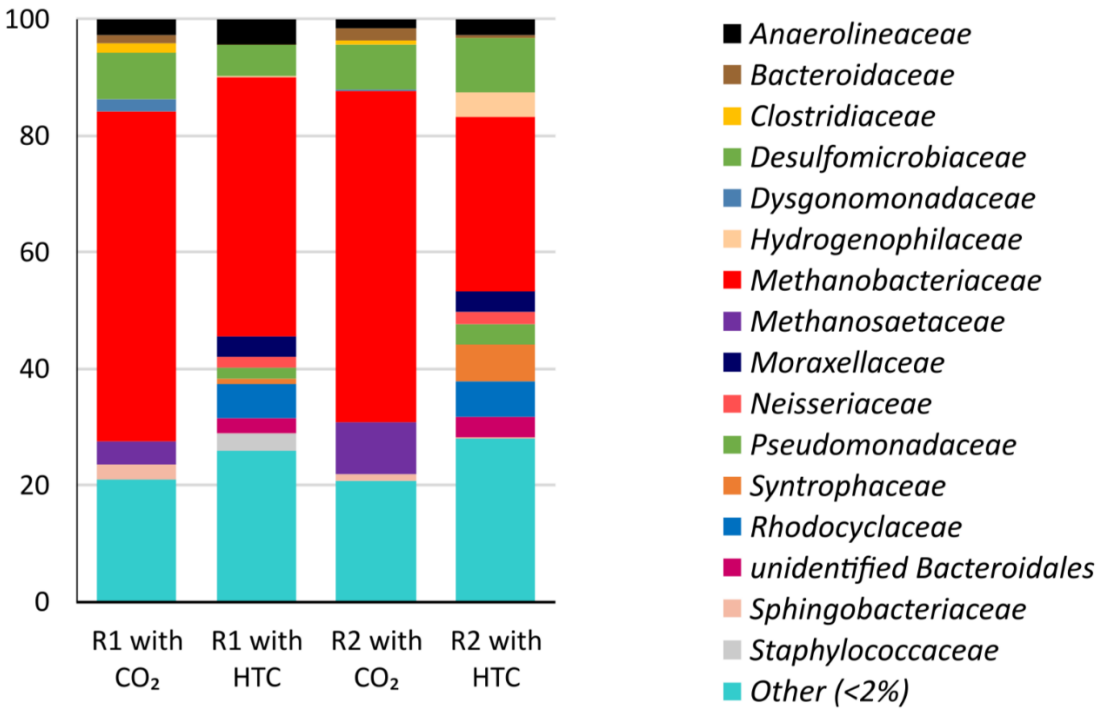

Figure 8.8. *Relative abundance of microorganisms fed with CO_2 and with HTC. Groups that did not reach at least 2% were grouped in the category "Other"*

Regarding Bacteria, their diversity was greater than that of Archaea. The *Desulfomicrobiaceae* family, capable of electrotrophic hydrogen production [40], occupied a preeminent position in terms of relative abundance regardless of the gas fed. Interestingly *Rhodocyclaceae*, *Sphingobacteriaceae* and *Anaerolineaceae* families —all of them microorganisms also capable of using the electrons arriving at the cathode to catalyse reductive process such as H_2 formation [22,25,41–44]— increased its proportion in the presence of HTC off-gas. In addition, other microorganisms with a less clear role in methane production such as *Moraxellaceae* (previously described as electrotrophic bacteria in cathodic environments [45,46]), *Neisseriaceae*, *Pseudomonadacea* (electrotrophic denitrifier [47] and oxygen scavenger in biocathodes [48]) and

Synthrophaceae also increased their relative proportion in the presence of HTC. This observation might be revealing that CO is inducing a shift in the bacterial communities that results in the selection of those families directly involved in electrotrophic reductive processes. As these bacteria need to be in close contact with the electrode, it can be hypothesised that Archaea ─that relay on the hydrogen generated by Bacteria─ might be forming a protective biofilm above them that alleviate the potential impact of CO.

8.4. Conclusions

This chapter demonstrates the technical feasibility of converting HTC off-gas into methane through EM. Results reveal that although this gas severely affects both current density and methane production, it allows the production of biogas with up to 70% of methane content. HTC off-gas also had a negative impact on the cathodic microbial communities, especially on the archaeal family *Methanomicrobiaceae* that uses hydrogen to produce methane. Although feeding HTC off-gas also resulted in a decrease in the total number of gene copies of Bacteria, the impact was less pronounced, probably because the Archaea form a protective biofilm. Finally, it was hypothesised that the CO present in the HTC could be responsible for this biological inhibition, although its eventual conversion to methane could also lead to higher coulombic efficiencies.

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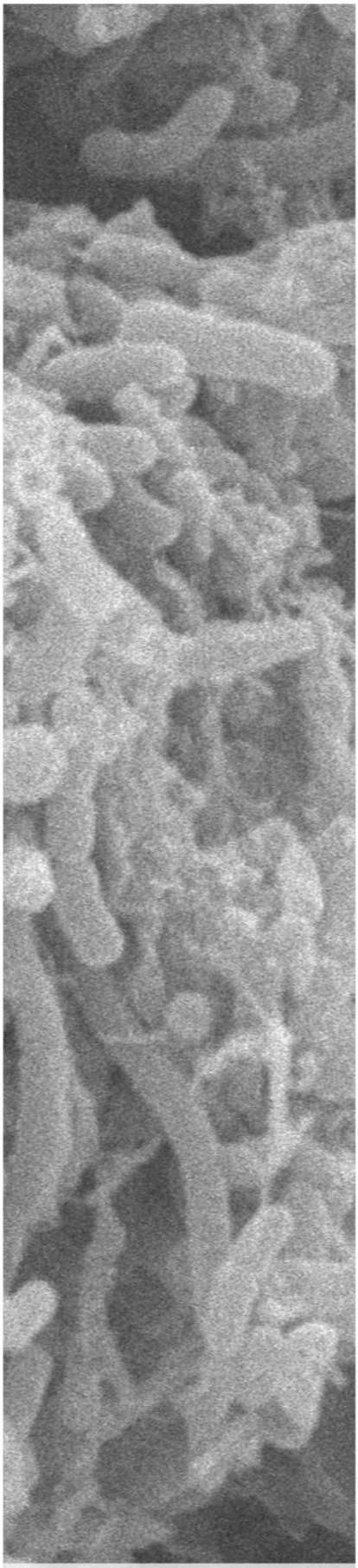

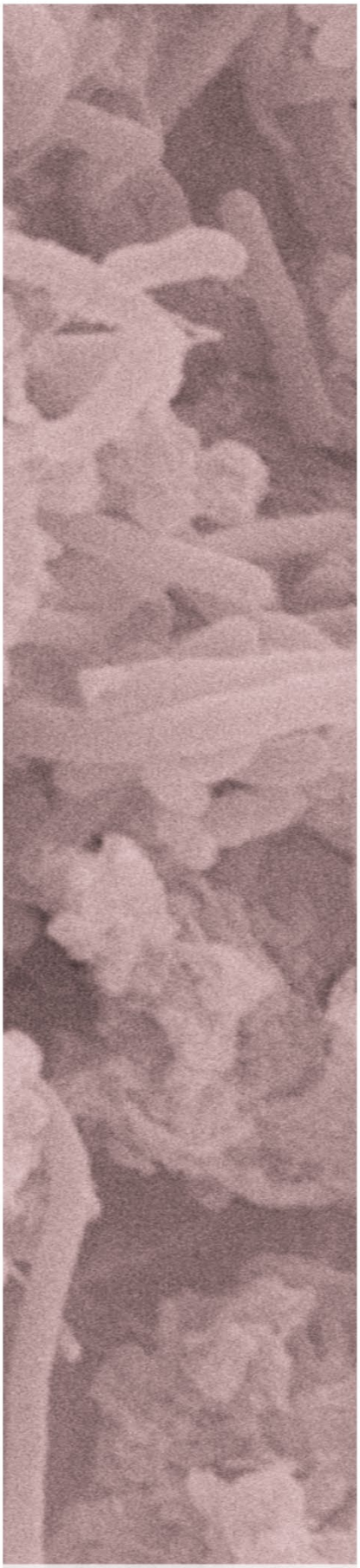

CHAPTER 9

CONCLUSIONS AND FUTURE PERSPECTIVES

9.1. Conclusions

Biogas upgrading by means of electromethanogenesis (EM) has aroused a notable interest among scientist and engineers and it is taking its first steps towards its future practical application. This thesis aims to gain insight into the nature of some key challenges that need to be overcome before this technology can be scaled up. Specifically, this thesis focuses on studying the impact that power fluctuations, temperature, pH and the presence of impurities of the gas fed to the biocathode has on the EM performance and on the selection of cathode microorganisms. The work that has been here presented has led to the following conclusions:

- Results confirm that H_2 is the main intermediate between electron uptake and methane formation from CO_2. Thus, EM seems to occur in two-step process that involves electrotrophic H_2 generation (led by bacteria such as *Desulfovibrio* sp.) and methanogenic H_2 consumption (led by hydrogenotrophic archaea such as *Methanobacterium* sp.).
- Power interruptions of up to 96 h do not have a significant impact on EM rates. However, when power gaps occur in the absence of externally added H_2, current is less efficiently used in the production of methane once the power supply is reconnected.
- Current density and methane production rate became less reproducible (larger variations between replicates) in the absence of hydrogen during power-gaps, which can be attributed to a disruption in the metabolic routes or a shift in the microbial populations caused by a prolonged lack of electron donors.

- Electrotrophic communities are more robust that the methanogenic communities to medium-lower temperatures. Current density in methanogenic biocathodes can be as high as 2.5 $A \cdot m^{-2}$, showing little dependence on temperature. In contrast, methane productivity is highly dependent on this parameter.
- A more acidic condition (pH 5.5) leads to faster start-up and faster product formation in MES/EM reactors but pH did not seem to have any impact on product selectivity.
- Production efficiency increased with acidity (pH 4.5) due to increased H_2 production and acid tolerance of *Methanobacterium* sp.
- It is technically feasible to convert a real substrate such as HTC off-gas into methane through EM although this gas severely affects both, current density and methane production.
- CO present in the HTC could be responsible for biological inhibition of biocathode population, although its eventual conversion to methane could also lead to higher coulombic efficiencies.

Overall, these results show that CO_2 streams upgrading by EM is possible and that it is certainly resilient to the impacts of current fluctuations, temperature or pH variations. The compounds that accompany CO_2 in the biogas used as a substrate are key and have an important impact on the current density and production rates.

*9.1bis. Conclusiones (*Spanish*)*

La mejora del biogás mediante la electrometanogénesis (EM) ha despertado un notable interés entre científicos e ingenieros y está dando sus primeros pasos hacia su futura aplicación práctica. Esta tesis pretende conocer la naturaleza de algunos retos clave que deben superarse antes de que esta tecnología pueda ser escalada. En concreto, esta tesis se centra en el estudio del impacto que las fluctuaciones de suministro eléctrico, la temperatura, el pH y la presencia de impurezas del gas alimentado al biocátodo tienen en el rendimiento del EM y en la selección de los microorganismos del cátodo. El trabajo que aquí se ha presentado ha llevado a las siguientes conclusiones:

- *Los resultados confirman que el H_2 es el principal intermediario entre la captación de electrones y la formación de metano a partir del CO_2. Así, la EM parece ocurrir en un proceso de dos pasos que implica la generación electrotrófica de H_2 (liderada por bacterias como* Desulfovibrio *sp.) y el consumo metanogénico de H_2 (liderado por arqueas hidrogenotróficas como* Methanobacterium *sp.).*
- *Las interrupciones de corriente de hasta 96 horas no tienen un impacto significativo en las tasas de EM. Sin embargo, cuando se producen interrupciones en ausencia de H_2 añadido externamente, la corriente se utiliza de forma menos eficiente en la producción de metano una vez que se vuelve a reconectar el suministro de energía.*
- *La densidad de corriente y la tasa de producción de metano son menos reproducibles (mayores variaciones entre réplicas) en ausencia de H_2 durante los cortes de energía, lo que puede atribuirse a una interrupción de las rutas metabólicas o a un cambio en las poblaciones*

microbianas causado por una falta prolongada de donantes de electrones.

- *Las comunidades electrotróficas son más robustas que las metanogénicas a temperaturas medias y bajas. La densidad de corriente en los biocátodos metanogénicos puede ser de hasta 2,5 $A \cdot m^{-2}$, mostrando poca dependencia de la temperatura. Por el contrario, la productividad de metano es altamente dependiente de este parámetro.*
- *Una condición más ácida (pH 5,5) conduce a un arranque más rápido y a una formación más rápida del producto en los reactores MES/EM, pero el pH no parece tener ningún impacto en la selectividad del producto.*
- *La eficiencia de la producción aumentó con la acidez (pH 4,5) debido a la mayor producción de H_2 y a la tolerancia al ácido de* Methanobacterium *sp.*
- *Es técnicamente factible convertir un sustrato real como el gas de salida del HTC en metano a través de la EM, aunque este gas afecta severamente tanto a la densidad de corriente como a la producción de metano.*
- *El CO presente en el HTC podría ser responsable de la inhibición biológica de la población del biocátodo, aunque su eventual conversión en metano también podría conducir a mayores eficiencias coulómbicas.*

En general, estos resultados muestran que la mejora del biogás mediante EM es posible y que es ciertamente resistente a los impactos de las fluctuaciones

de la corriente, la temperatura o las variaciones del pH. Los compuestos que acompañan al CO_2 en el biogás utilizado como sustrato son clave y tienen un impacto importante en la densidad de corriente y en las tasas de producción.

9.1. Future perspectives

The aim of this thesis was to contribute to the use of EM technology outside of the laboratory, and according to the results obtained, we can think that EM is closer to it. However, there is still a long way to go due to the challenges that remain unsolved.

Given the results obtained using HTC off-gas as a substrate, it is evident that other types of biogas will need to be tested, in order to discover how traces of non-degraded compounds can affect the EM process, or if the process itself can serve as a bioremediation process.

Electrodes geometry and electrodes material lay at the very core of EM. The possibilities offered by techniques that improve electrical capacity such as graphene electrodeposition or the design of electrodes of different materials and shapes adapted to the 3D printing potentialities, can increase the opportunities for this technology to become more accessible for industrial application.

Another important aspect of EM that has not been addressed yet is reactor architecture and reactor configuration. The H-type reactors used in this thesis provide and suitable, versatile, and easy-to-use platform for lab-experiments. However, they are far from optimal for use in real life applications. The scale up and optimization EM reactors would have to face the challenges typically found

in electrochemical systems (increased overpotentials with size, electrodes and membrane fouling and deterioration, etc) and those of more conventional bioreactors such as mass transfer limitations, and problems related with stirring and hydrodynamics.

Finally, another area where there are still gaps in knowledge is the microbiology of the biocathode. Although considerable efforts have been made in recent years, the biological part remains one of the mysteries of BES in general. The opportunities offered by recent advances in bioengineering could be a turning point in the operability of these systems, developing organisms that are resilient to those factors that usually have a negative impact on performance while enhancing reactions that are not yet efficient enough,

APPENDIXES

APPENDIX 1. Chapter 5: supporting information

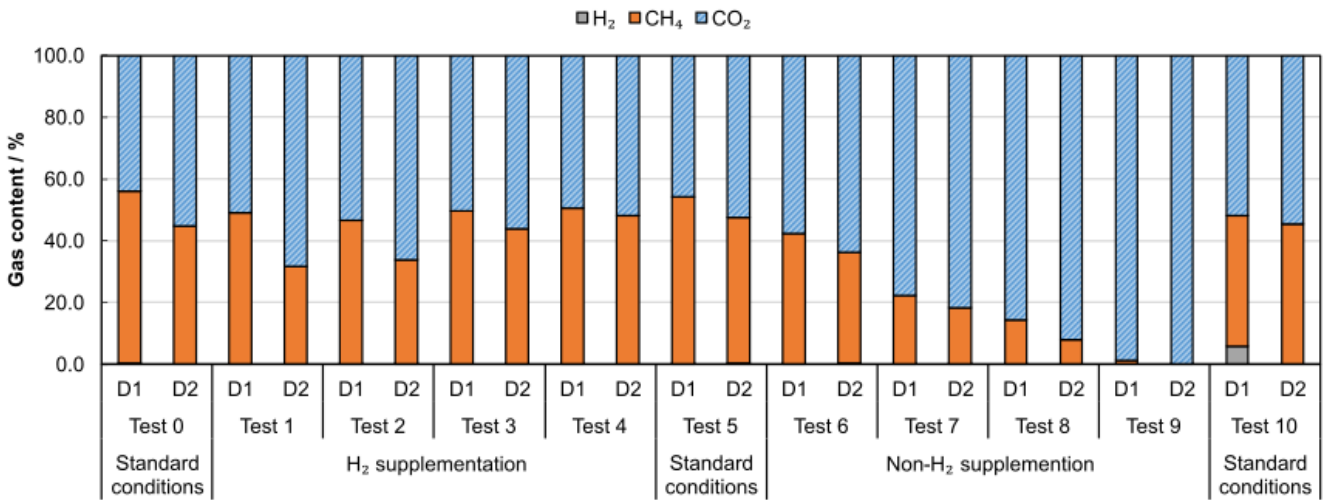

Figure S5.1. *Gas content*

APPENDIX 2. Chapter 6: supporting information

***Table S6.1**. 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%

APPENDIX 3. Chapter 7: supporting information

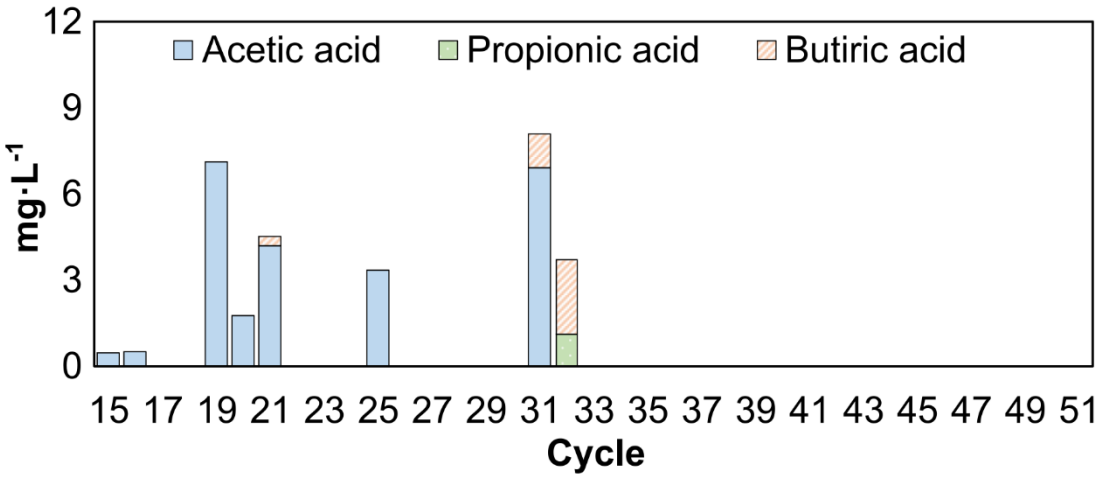

***Figure S7.1**. Production of volatile fatty acids (in mg·L^{-1}) in reactor P7 at pH 7.5 from cycles 15 to 51.*

APPENDIX 4. Chapter 8: supporting information

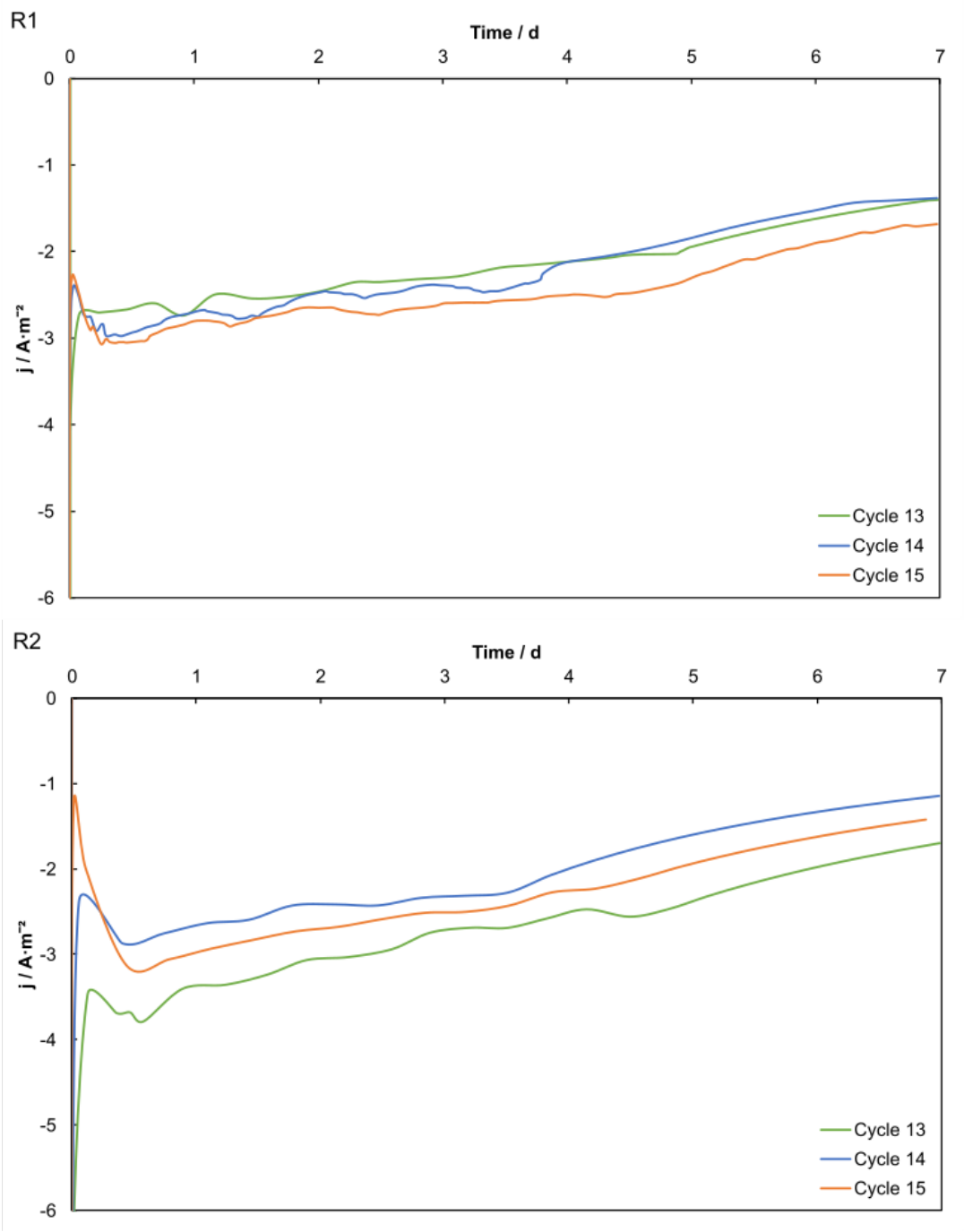

Figure S8.1. *Current density profiles for the last 3 cycles of startup period for reactors R1(top) and R2 (bottom).*

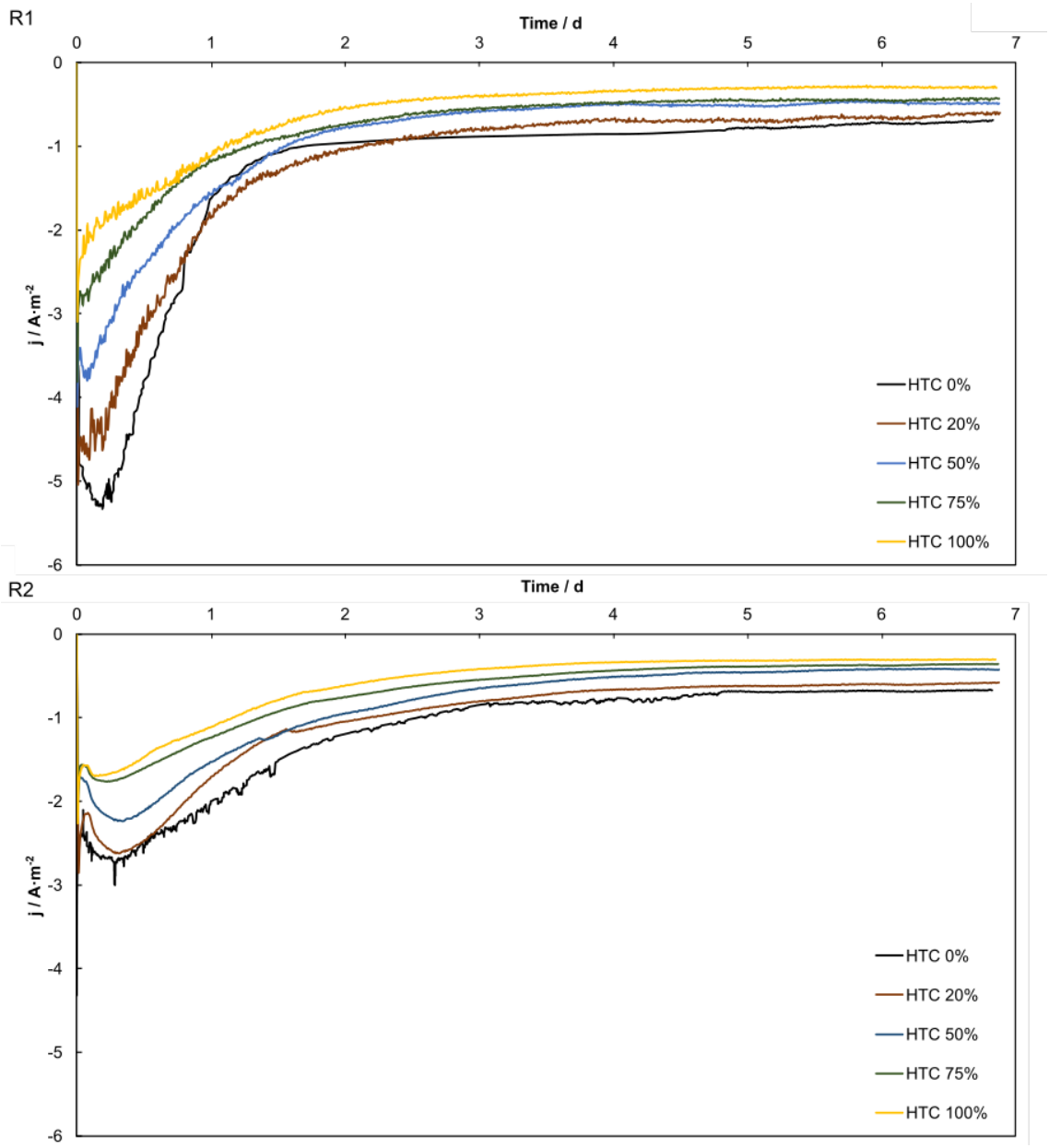

Figure S8.2. *Current density profiles obtained during the experimental phase for reactors R1(top) and R2 (bottom) at different HTC concentrations.*