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Physiological and Operation Strategies for Optimizing *Geobacter*–based Electrochemical Systems

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TESIS DOCTORAL

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Physiological and Operation Strategies for Optimizing *Geobacter*-based Electrochemical Systems

Memoria presentada para optar al título de Doctor por la Universidad de Alcalá por :

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A mis padres y a mi hermano

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ABBREVIATIONS AND UNITS

ABC: ATP-binding cassette

AEM: Anion Exchange Membrane

ATP: Adenosine Triphosphate

BES: Bioelectrochemical System

CA: Chronoamperometry

cAMP: cyclic Adenosine Monophosphate

CE: Counter Electrode

CEM: Cation Exchange Membrane

COD: Chemical Oxygen Demand

CV: Cyclic Voltammetry

d: Days

DEET: Direct Extracellular Electron Transfer

DGC: Diguanylate Cyclase

DIET: Direct Interspecies Electron Transfer

DNA: Desoxirribonucleic Acid

e^-: Electrons

E: Potential

E^0: Standard potential

EC: Electric Conductivity

ED-MDC: Electrodyalisis Microbial Desalination Cell

ED: Electrodyalisis

EDTA: Ethylenediaminetetraacetic Acid

EET: Extracellular Electron Transfer

E_i: Initial potential of the potential sweep in a cyclic voltammetry

E_l': Limit potential for the inversion of the sweep in cyclic voltammetry

E_p: Peak potential

E_{pa}: Anodic peak potential

E_{pc}: Cathodic peak potential

EPS: Extracellular Polimeric Substances

F: Faraday constant

FO: Forward Osmosis

FWM: Fresh Water Medium

h: Hours

HSP: Heat Shock Proteins

I: Current

I_p: Peak Current

I_{pa}: Anodic peak current

I_{pc}: Cathodic peak current

j: Current density

J_{max}: Maximun current density

MDC: Microbial Desalination Cell

MEC: Microbial Electrolysis Cell

MERC: Microbial Electroremediating Cell

MES: Microbial Electrosynthesis

MET: Microbial Electrochemical Technologies

MFC: Microbial Fuel Cell

MSC: Microbial Short Circuited

NAD^+: Nicotinamide Adenine Dinucleotide (oxidised)

NADH: Nicotinamide Adenine Dinucleotide (reduced)

NERS: Nanoparticle-enhanced Raman Spectroscopy

OCP: Open Circuit Potential

OMC: Outer Membrane Cytochrome

photoMFC: photomicrobial fuel cell

Ppc: Periplasmic Cytochrome

Q: Charge

RE: Reference Electrode

RMDC: Ion-exchange Resin Coupled Microbial Desalination Cell

rMDC: Recirculation Microbial Desalination Cell

RO: Reverse Osmosis

s: Seconds

SEM: Scanning Electron Microscopy

SHE: Standard Hydrogen Electrode

sMDC: Stacked Microbial Desalination Cell

sMFC: Sediment Microbial Fuell Cell

T: Temperature

t: Time

TCA: Tricarboxylic acid cycle

TEA: Terminal Electron Acceptor

V: Volts

V_{an}: Anolyte Volume

V_{cat}: Catholyte Volume

V_{des}: Saline Stream Volume

WE: Working Electrode

ΔomcB: knock-out OmcB mutant strain

Summary/Resumen

SUMMARY

The discovery of bacteria (e.g. *Geobacter sulfurreducens*) able to interact at redox scale with electrical conductive materials triggered the development of a new platform: the so-called Microbial Electrochemical Technologies (METs). METs were, from the very beginning, strongly aimed to convert the large amount of chemical energy present in waste into a useful electrical current. However, during the last decade, a myriad of new MET-based applications have emerged in the field of bioremediation, biosensing, water desalination and bioelectrosynthesis among many others.

This PhD thesis explores new strategies for enhancing METs capacities by analysing parameters operating at both micro-scale and pre-pilot scale. The thesis is organized in two main parts, followed by a general discussion and conclusions section. Each part consists of one introductory chapter, which constitutes the state of the art, followed by the methodology and the experimental chapters.

In METs, regarding to the micro-scale issue, *G.sulfurreducens* is able to transfer the electrons from acetate to an electrode in a process known as Direct Extracellular Electron Transfer (DEET). DEET is the core of the energy harvesting process in microbial electrochemistry; therefore it becomes one of the key points in the optimization of METs performance. Most of the efforts to elucidate the key players and their respective role in DEET have been focused either in genetic engineering or in the optimization of technical parameters of METs. Nevertheless, important issues like the constitutive electroactivity of microbes under an electrode-free scenario has received less attention. In **Chapter 2,** we have explored the use of a novel protocol based on salt stress for enhancing the

electroactivity of planktonic *G.sulfurreducens*. Evidences of the contribution of structures as the extracellular polymeric substances (EPS) were also reported. Actually, these **EPS are proposed to be an electroactive network able to electrically plug planktonic *G. sulfurreducens* cells into electrodes.**

The physiological state of the microbial inoculum is key not only for harvesting current, but also for reducing operation periods in METs. To explore this hypothesis, we devoted the **Chapter 4 to test a filter-press based bioreactor for treating both acetate-supplemented synthetic wastewater and real urban wastewater.** This proof-of-concept pre-pilot treatment included a microbial electrolysis cell (MEC) followed in time by a microbial fuel cell (MFC) to finally generate electrical current. The effective removal of acetate suggests a potential use of this modular technology for treating acetogenic wastewater where *Geobacter sulfurreducens* outcompetes other organisms. Two *G. sulfurreducens* inocula, showing different physiology, were analysed in order to determine its influence on the growth of the electroactive biofilm. Interestingly, the physiology acquired under electron acceptor limitation in a chemostat, showed a significant electroactive response that reduced the start-up period of the system in almost 20-fold. In addition, **we successfully identified OmcB as the cytochrome responsible for this high electroactive phenotype**.

In METs platform, Microbial Fuel Cells (MFC) are the main devices that exploit electroactive microorganisms to harvest electrical energy from organic matter. In this context, a promising MFC-derivate technology is Microbial Desalination Cell (MDC), a system able to couple wastewater treatment, energy generation and

desalination in one single device. As a derivate technology, the start-up protocol typically entails the operation as MFC before desalinating under MDC operation. In **Chapter 5 we have presented an optimized procedure based on merging *Geobacter sulfurreducens* electrochemistry with electrodialysis.** The results obtained validate the pre-pilot system operation and the protocol for reducing the start-up period. This start-up protocol is not only optimized in time but also simplified in operation procedures making it a more feasible strategy for future scaling-up either as a single process or as a pre-treatment method combined with other well established desalination technologies such as reverse osmosis (RO) or reverse electrodialysis.

This thesis ends with a final section, which presents a general discussion together with the main conclusions of the experimental work.

RESUMEN

El descubrimiento de algunas bacterias, como *Geobacter sulfurreducens,* capaces de interaccionar a nivel redox con materiales conductores de la electricidad ha permitido el desarrollo de una nueva plataforma de tecnologías denominadas electroquímicas microbianas o METs (Microbial Electrochemical Technologies). Las METs se concibieron en sus inicios para la recuperación, en forma de corriente eléctrica, de la energía química almacenada en la materia orgánica de distintos residuos. Sin embargo, la extensa investigación a la que han sido sometidas en la última década ha generado numerosas aplicaciones en el campo de la biorecuperación de suelos y agua, el diseño de biosensores, la desalación de agua, o la electro síntesis orgánica..

Esta tesis doctoral explora nuevas estrategias para mejorar el funcionamiento de las METs, mediante el análisis de parámetros tanto a escala micro como a escala pre-piloto. La tesis se ha organizado en dos bloques prinicpales, seguidos de una sección que incluye una discusión general y unas conclusiones. Cada bloque consta de un capítulo introductorio, que incluye el estado del arte, seguido de capítulos metodológicos y experimentales.

Si analizamos las METs, a escala micro, *G.sulfurreducens* es capaz de transferir los electrones de la oxidación del acetato a un electrodo en un proceso conocido como transferencia extracelular de electrones directa (DEET, Direct Extracellular Electron Transfer). DEET es el proceso clave dentro de esta conversión electroquímica de residuos en energía, por lo tanto, se convierte en uno de los puntos clave para lograr la optimización de estos dispositivos. Muchos han sido los esfuerzos por esclarecer tanto los participantes en el proceso

de DEET como sus respectivas funciones en el mismo.. Con frecuencia, las estrategias planteadas se centran tanto en la manipulación genética del microorganismos como en la optimización de los aspectos técnicos de las METs. Sin embargo, poca atención ha recibido el estudio de electroactividad constitutiva de estos microorganismos en sistemas libres de electrodos. Así, en el **capítulo 2**, presentamos un protocolo basado en utilizar el stress salino como factor para estimular la electroactividad de células planctónicas de *G.sulfurreducens*. Con este análisis se obtuvieron evidencias de la contribución de sustancias poliméricas extracelulares (EPS, Extracellular Polymeric Substances), en el proceso de DEET. De esta forma, en esta tesis se propone a **la matriz de EPS como una red electroactiva capaz de conectar a las células planctónicas de *G. sulfurreducens* con electrodos.**

Nuestros estudios revelan como el estado fisiológico del inóculo bacteriano es clave para el funcionamiento de las METs, no sólo en cuanto a la producción de corriente, sino también a la reducción de los períodos de operación. Para demostrarlo, en el **capítulo 4 se utilizó un prototipo tipo filtro prensa, a escala pre-piloto, en el que se ensayaron aguas residuales reales y aguas sintéticas suplidas con acetato.** El tratamiento a escala pre-piloto incluyó el uso de una celda de electrólisis microbiana (MEC) seguida, en el tiempo, de una celda de combustible microbiana (MFC). La eliminación eficiente de acetato sugiere el uso de este sistema modular para el tratamiento de aguas acetogénicas donde *G. sulfurreducens* desplazaría a otros microorganismos. Además se analizó el impacto en la generación del biofilm electroactivo tras utilizar dos inóculos de *G.sulfurreducens* con distinta fisiología.. Se pudo demostrar como la fisiología adquirida en un quimiostato bajo

limitación de aceptor de electrones resultó óptima para reducir, en casi 20 veces, el tiempo de arranque del sistema. **Además identificamos el papel del citocromo C OmcB en el fenotipo electroactivo de estas células planctónicas.**

En la plataforma de las METs, las celdas de combustible microbianas (MFC, Microbial Fuel Cell) son los principales dispositivos que explotan la capacidad de los microorganismos electroactivos para generar energía eléctrica. En este contexto, una tecnología prometedora, derivada de las MFC, son las celdas microbianas de desalinización (MDC, Microbial Desalination Cell). Las MDC son dispositivos capaces de tratar agua residual y desalinizar agua salobre. Como tecnología derivada, el protocolo convencional de puesta en marcha implica la operación del sistema como MFC antes de poder desalinizar. Así, en el **capítulo 5 se presenta un protocolo optimizado basado en integrar la electroquímica de *Geobacter sulfurreducens* en una electrodiálisis convencional con el objeto de reducir los tiempos de arranque**. Tanto el protocolo de puesta en marcha como el diseño del sistema propuesto se confirman como una tecnología de bajo consumo, viable y escalable para poder operar de forma autónoma, como pre-tratamiento combinada con otras tecnologías de desalinización como la ósmosis reversa.

Por último, la tesis incluye una sección final que recoge una discusión general junto a las principales conclusiones de nuestro estudio.

Thesis Context, Work Hypothesis and Objectives

THESIS CONTEXT

The present PhD thesis was developed into the frame of two research projects, Bacwire and IISIS, both focused on applying microbial electrochemical systems for treating wastewater.

Bacwire (Bacterial Wiring for Energy Conversion and Bioremediation) was a project funded by the European Commission under the 7th Framework Program. It was formed by a consortium of five research institutions: Electrochemistry group of Liverpool University (United Kingdom), Electrochemistry Institute of Alicante University (Spain), Group of electrochemical Nanoscience of Bern University (Switzerland), Research Institute in Materials Science and Technology (Argentina) and Bioelectrogenesis Group of the Alcalá University; and the electrochemical engineering company ElectroCELL. The aim of the project was to develop a practical and optimized microbial fuel cell for the simultaneous cogeneration of energy and bioremediation of wastewater using electroactive bacteria. The results presented in this thesis (Chapter 4) were related to some of the objectives aimed in Bacwire.

IISIS (Integrated Research on Sustainable Islands) was a project funded by the INNPRONTA program of CDTI (Spanish Economy and Competitiveness Ministry). Mainly private companies from the water & civil engineering sector like FCC and Aqualia, together with some public research institutions, among them Imdea Water, formed IISIS consortium. The project was focused on the concept of artificial islands able to operate in decentralized and sustainable way, so all urban necessities should be covered while minimizing the environmental impact. Therefore, the project covered

four essential areas: energy, water and environment, building and integration. Our scientific contribution was carried out in the water research area by designing and constructing a microbial electrochemical system for coupling wastewater treatment and desalination (Chapter 5).

WORK HYPOTHESIS AND OBJECTIVES

Since the unprecedented boom on electroactive microorganism's research, the water sector has shown high expectations on some of their applications, like e.g. Microbial Fuel Cells (MFC) and Microbial Desalination Cells (MDC). The goal of MFC is to harvest electrical energy from the microbial metabolism during wastewater treatment, while MDC is fully devoted to perform, simultaneously, wastewater treatment and desalination at zero operation cost. Nowadays, despite the huge information and advances achieved in these fields, there are still many limitations that impede not just device operating but also system scaling-up: e.g. internal resistance, non-favourable cathodic reactions but also non-optimal physiological conditions of electroactive bacteria.

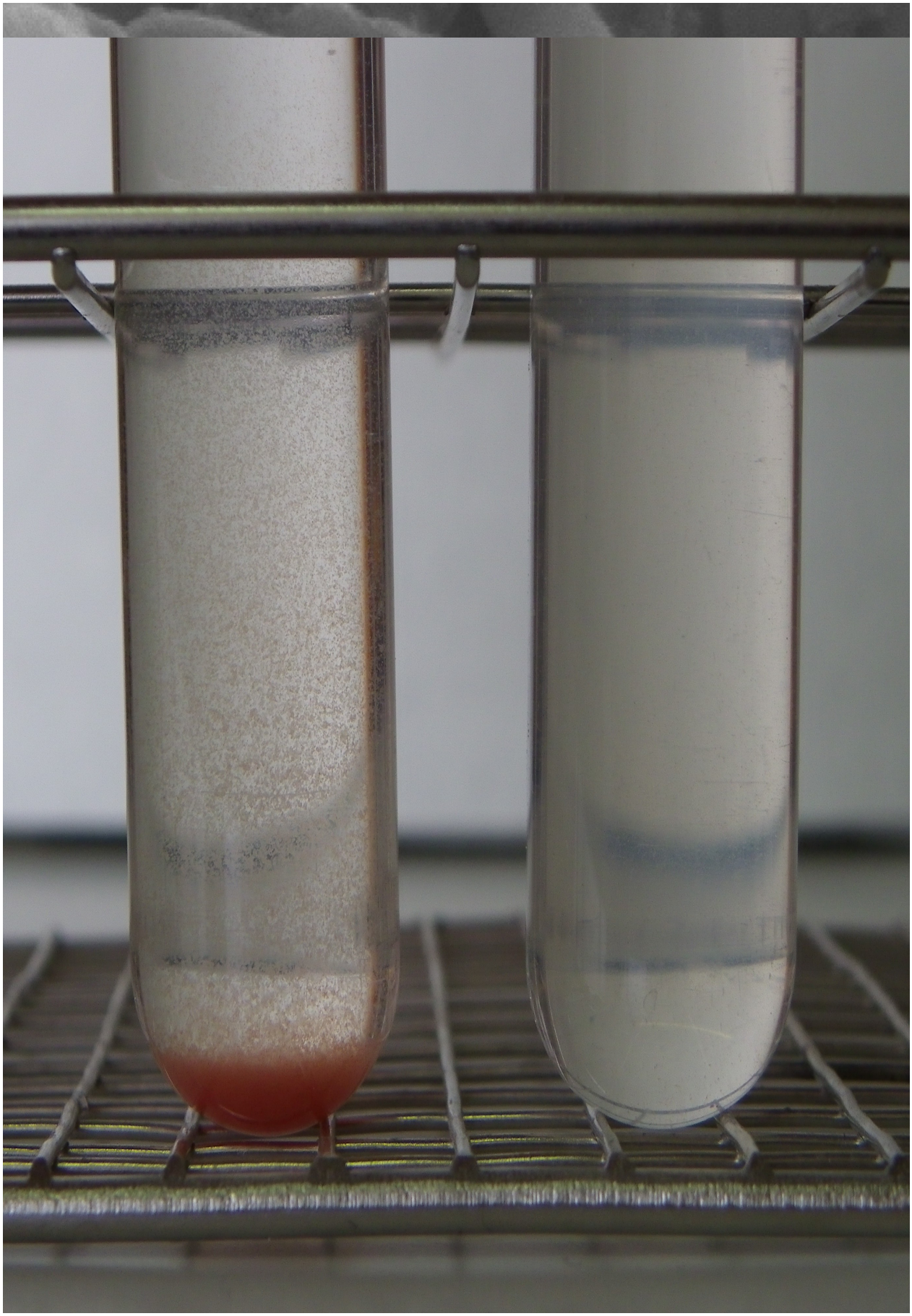
Electroactive microorganisms are indeed key in these innovative electrochemical technologies and, interestingly, most of the research has been so far focused on electroactive biofilms. In spite of this trend, we hypothesized that exploring the physiology of a planktonic electroactive, instead of biofilm, would generate unexpected electroactive physiological response (Chapter 2) with a potential application in Microbial Electrochemical Technologies.

In this context, we established a number of objectives with the aim of testing our main hypothesis.

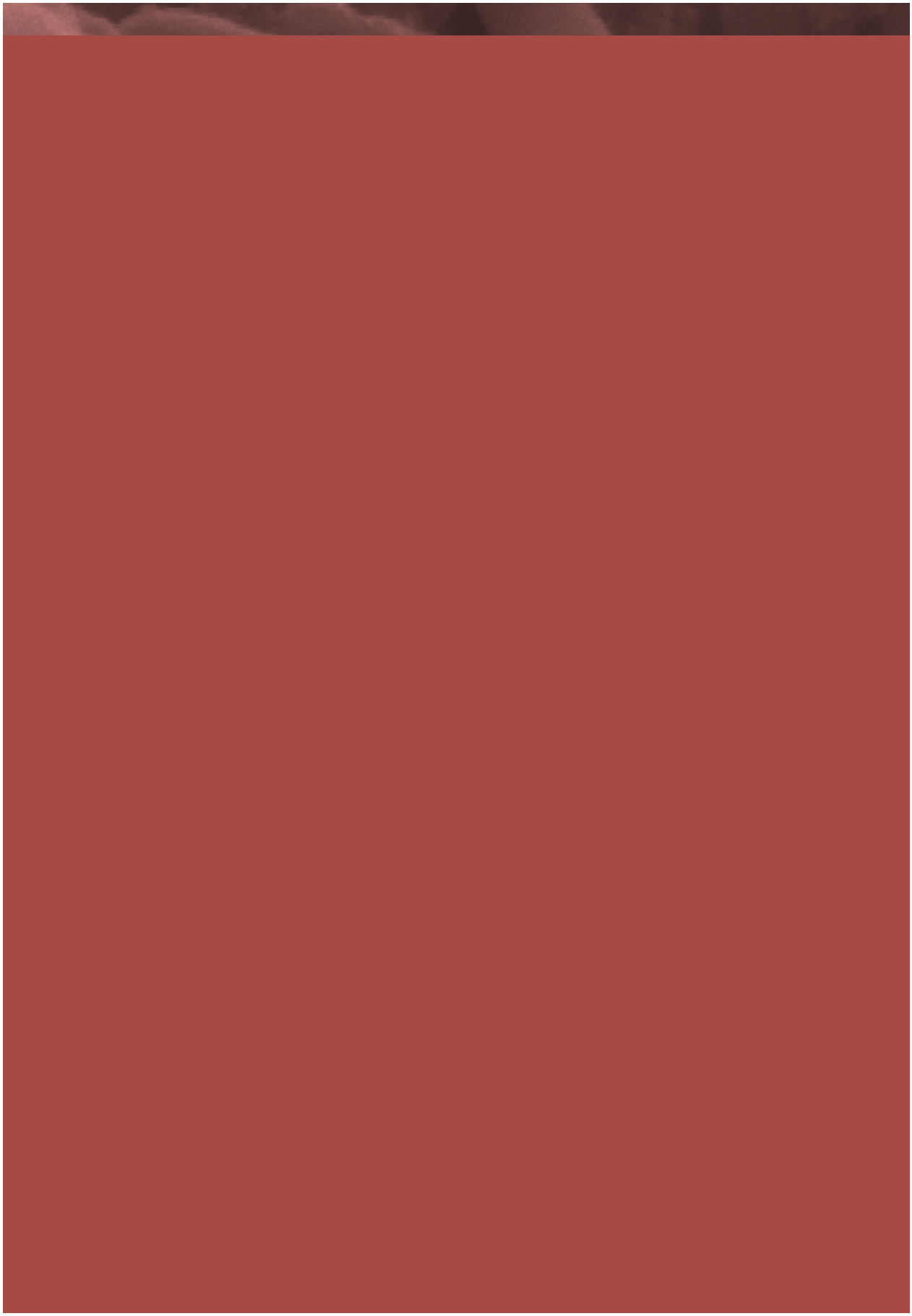
- Objective 1. To achieve electroactive planktonic cells of *Geobacter sulfurreducens* under saline stress.

- Objective 2. To achieve optimal electroactive planktonic cells of *Geobacter sulfurreducens* by culturing them in a chemostat under different redox conditions. The cells will be tested in a pre-pilot scale MFC for generating electrical current from acetogenic wastewater.

- Objective 3. To set-up a pre-pilot microbial desalination cell based on the electroactive bacteria *Geobacter sulfurreducens* for coupling biodegradation of acetogenic wastewater and desalination.



Part I. Electrochemistry and Living Cells



Chapter 1

Introduction to Electroactivity

Electroactivity and Living Cells: discovery and concept

The concept of *bioelectricity* was strongly advocated and experimentally supported in the animal field for the first time by Luigi Galvani (1737-1798) in his paper *De viribus electricitatis in motu musculari* (1791), which is considered the seed of electrophysiology. His assays, with frog nerve-muscle preparations, lead him to assume that the nerve and the muscle would be charged with animal electricity, which produces contraction when contacted by metals. Ones like H. Cavendish (1731-1810) supported the idea of an electric current generation by the own animal meanwhile others like Alessandro Volta (1745-1827) strongly rejected the idea of *bioelectricity.* Later on, Alexander von Humboldt (1760-1859) showed that two different phenomena do indeed occur: an electric current generated by the metallic contacts, but also a true intrinsic animal electricity production. Since then, many are the phenomenological aspects which bioelectricity is related to, like membrane potentials, reception and transmission of excitation between cells, synaptic potential, etc. Even to the electricity-generating ability that has some aquatic creatures such as the electric eel and the black ghost knife fish. (Molecular Basis and Thermodynamics of Bioelectrogenesis).

Nevertheless, the *bioelectricity* concept, which this thesis is based on, is different from the previous described. Since unicellular forms of bacteria are the responsible of generating the electrical current. In this case, the microbial electrochemical phenomenon consists in the capacity of certain microorganisms to transfer the electrons from their metabolism to a terminal electron acceptor (TEA) characterized for being extracellular and insoluble. With the

discovery of this kind of microorganisms named as electrogenic or electroactive, we took advantage of their natural capacity by replacing their TEA by a polarized electrically conductive material (electrode) in order to harvest the electrons of their metabolism as electric current.

Respiration in bacteria

In prokaryotes (no fermentative) respiration, the cell oxidizes the substrate (organic matter) and transfers the electrons in form of NADH (reduced nicotinamide adenine dinucleotide) to several multiproteic complexes associated to the inner cellular membrane, in order to generate accessible energy. These complexes compose the electron transfer chain; where the electrons are transported through it while protons are moved across the membrane generating a transmembrane gradient. Eventually, protons return into the cell through the ATPase complex, allowing ATP (adenosine try phosphate) synthesis, and electrons are transferred to a suitable terminal electron acceptor (TEA) (White, 2007). The redox potential of this TEA determines the energy available for cell growth (Mathews et al., 2002). On top of that, the nature of the TEA makes bacterial respiration to occur at inner membrane (soluble TEA) or at extracellular environment (insoluble TEA).

Soluble terminal electron acceptor are known from the very beginning of microbiology history. Among them, oxygen is the model TEA molecule since it is a membrane diffusible gas and a highly oxidizing agent and, therefore, an excellent electron acceptor in aerobic respiration (Figure 1). However, in environments devoid of

oxygen, like for example marine sediments, anaerobes require alternatives TEAs such as sulphate or nitrate.

When the TEA is an insoluble molecule not able to cross the cellular membrane, like iron or manganese oxides, a strategy to connect internal respiratory chain to the external electron acceptor is necessary.

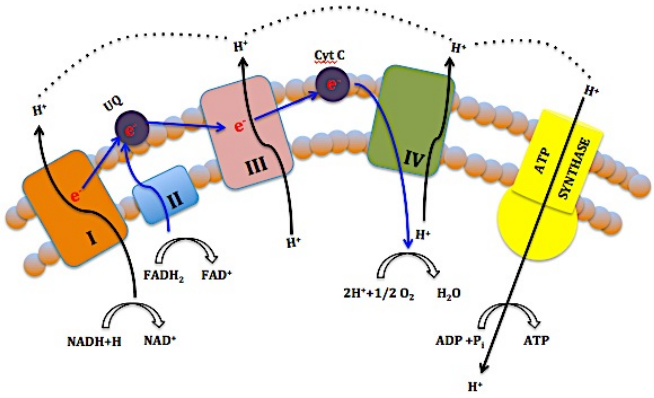


Figure 1. Electron transport chain composed by: I, NADH deshydrogenase; II, Succinate deshydrogenase; III, Cyrochrome reductase; IV, Cytochrome oxidase. In a standard aerobic respiring organism nicotinamide adeninde dinucleotide (NADH) or flavin adenine dinucleotide (FADH) coming from oxidative metabolism feed the respiratory electron transport chain at both complex I and II, respectively. The complex III and IV reduction led to proton expulsion to the periplasm. Electrons keep moving through the transport chain driving by a redox potential difference until reaching the last complex, which reduce a terminal electron acceptor (oxygen). The protons propelled outside the cytoplasm come back inside through the ATPse complex, allowing ADP phosphorylation.

Iron reducing bacteria

In the absence of molecular oxygen, bacteria can use a variety of terminal electron acceptors for respiration such as hydrous ferric oxide, goethite, hematite, manganese and iron oxides for respiration.

The capacity of microorganisms to reduce metals as a cofactor

for the synthesis of macromolecules as proteins, it has been known for over a century. However, the existence of specialist bacteria that can use such activity to conserve energy for growth under anaerobic conditions, it is fairly recent. The first extracellular respiratory processes that were discovered were iron and manganese respiration in *Shewanella* and *Geobacter* species (Lovley and Phillips, 1988; Lovley et al., 1987; Myers and Nealson, 1988). Since then, extracellular respiration became an important focus for the work in Microbiology. The importance of these respiratory processes can be assessed considering that iron is the most abundant redox-active metal in today's Earth's crust. Consequently, several studies pointed out that respiratory iron reduction could massively contribute to the oxidation of organic carbon sources in a variety of anaerobic habitats (Bongoua-Devisme et al., 2013; Lovley, 1991; Thamdrup et al., 2000). Besides the relevance of biodegrading organic matter in anaerobic environments, iron reduction is important for the biodegradation of pollutants such as oil residues, landfill leachates, etc. in contaminated groundwater (Lovley et al., 2011). In polluted sediments, the microbial activity consumes the oxygen from contaminated areas leading to an anaerobic environment in which iron is usually the more abundant TEA (Lovley, 1991, 1997).

Extracellular electron transfer: How do microbes respire metals?

Unlike other metabolisms where the final electron acceptor is freely diffusible gas or readily soluble species, metal reducers face the challenge of transferring electrons to an extracellular insoluble form.

The extracellular electron transfer process entails the transport of electrons from the transport chain located in the inner membrane to cell exterior going through the periplasm and the outer membrane. This process is known as extracellular electron transfer (EET) (Hernandez and Newman, 2001). EET can be performed by two main electron transfer mechanisms that can be classified as direct or indirect. Both are not mutually exclusive and microorganisms may be capable of using them simultaneously. They are represented in Figure 2.

Indirect electron transfer

-Indirect electron transfer by reduced metabolic products:

This process takes place in fermentative microorganisms when convert glucose in metabolic products such as alcohols, acids or hydrogen. These products are secreted outside the cell and they reduce the extracellular TEA.

-Mediated electron transfer:

Another indirect strategy for the extracellular electron transfer is the use of redox mediators or electron shuttles. These compounds are reduced (accept electrons) in the inner transport chain and then diffuse towards the cell exterior where they are oxidised in the oxide surface of the TEA, before returning inside the cell in their oxidized-form. In this way, they become the insoluble TEA into an accessible electron acceptor (Figure 2B). This mechanism is widely spread and has been deeply studied in bacteria such as *Shewanella oneidensis, Geothrrix fermentans* (Marsili et al., 2008;

Nevin and Lovley, 2002) and some species of *Pseudomonas (*Hernandez et al., 2004*;* Rabaey et al., 2005).

The redox compounds are usually soluble molecules such as metal chelates (secreted by *Shewanella*) (Marsili et al., 2008) and phenazines (secreted by *Pseudomonas*) (Gescher and Kappler, 2012; Wang et al., 2010)(Figure 2A) that can be reversibly oxidized and reduced.

Direct electron transfer

There are some microorganisms, as the ones that belong to the family *Geobacteraceae*, that require the direct physical contact between themselves and the metallic oxide to be use as TEA (Bond and Lovley, 2003)(Figure 2C). As they do not depend on the synthesis of redox mediators for the respiration of non-soluble oxides (Bonanni et al., 2012a), they count on the presence of a mechanism that involves membrane-bound electron carriers that expand the electron transport chain from the inner membrane to extracellular environment (Bond and Lovley, 2003).

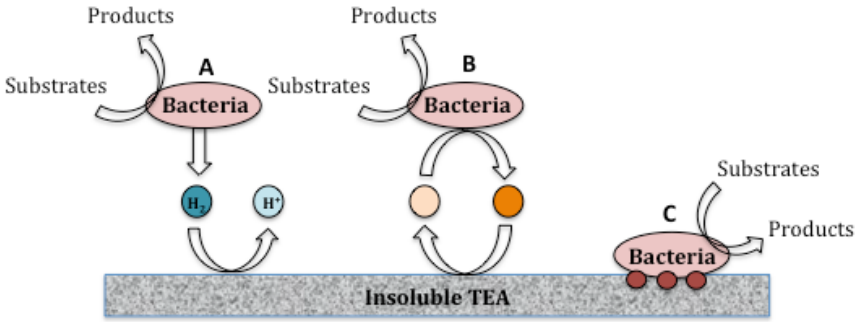


Figure 2. Electron transfer mechanisms. A: indirect electron transfer by metabolic products; B: mediated electron transfer; C: direct electron transfer by membrane bound proteins.

Extracellular electron transfer towards electrodes

Although iron oxides reduction was described in 1987 (Lovley et al., 1987), it took 14 years more to report electrodes as alternative TEA in the environment. In the key work of Reimers et al. (2001) a graphite electrode (the anode) was buried in the anoxic marine sediment and connected to a graphite cathode in the overlying aerobic water. In this way, they demonstrated that it was possible to harvest low-level power from natural, microbe established, voltage gradient at marine sediment-seawater interfaces.

Deeper studies on those energy-harvesting anodes revealed that they were enriched on microorganisms of the family *Geobacteraceae*. These microorganisms were able to conserve energy to support their growth by oxidizing organic compounds in the marine sediments with an electrode serving as the sole TEA (Bond, 2002). In the same work, these researchers demonstrated that pure cultures of the species *Geobacter sulfurreducens, Geobacter metallireducens and D. acetoxidans* inoculated into anaerobic devices, in which a graphite electrodes served as the sole electron acceptor and acetate or benzoate (i.e. for *G. metallireducens*) were the electron donor (Bond and Lovley, 2003), were capable to deliver their metabolic electrons to the electrode. These results indicated for the first time that electrode reduction could support bacteria growth.

Although the capacity of respiring electrodes is an unintended consequence of the ability of these bacteria to respire insoluble oxides, it has been demonstrated that the molecules involved in the EET process are different with these different TEAs (see below).

Geobacteraceae family

Among delta *Proteobacteria*, the first microorganisms shown to use iron oxides as TEA for energy conservation belong to *Geobacteraceae* family (Lovley et al., 1987). Moreover, in those environments where iron reduction is a favourable process, the most abundant microbial species were indeed from the genus *Geobacter* (Anderson et al., 2003; Holmes et al., 2007; Hori et al., 2010; Islam et al., 2004; Kerkhof et al., 2011, 2011; Roling et al., 2001; Rooney-Varga et al., 1999; Stein et al., 2001). Members of the *Geobacter* family are present in diverse sediments from aquifers polluted with oil, uranium o high concentrations of arsenic, chlorinated compounds, in methanogenic digesters, etc. (Lovley et al., 2011). *Geobacter* is actually able to promote the biodegradation of pollutants in those environments (Lovley et al., 2011). On top of the subsurface, *Geobacter* has been found in environments as different as wastewater (Aguirre et al., 2016, Submitted) or even gastrointestinal tract of ruminants (Guzman et al., 2015).

Due to the straightforward methods for isolating and culturing *Geobacter* species, more than 20 isolated species are currently available (Lovley et al., 2011). All of them have been classified as Gram-negative rod shape bacteria, able to oxidise organic acids of low molecular weight like acetate and to use iron oxides as TEA. They are also able to reduce others as Mn^{+4}, U^{+6}, S and humic substances (Lovley et al., 1996) while using ethanol or hydrogen as electron donors (Lovley et al., 2011). Regarding the genetic potential, ten genomes of *Geobacter* have been completely sequenced (Badalamenti and Bond, 2015; Lovley et al., 2011). The *Geobacter* capacity to oxidize acetate with iron oxides as TEA, makes *Geobacter*

an important player in the ecology and biogeochemistry of soils and sediments because acetate is the central intermediate in the anaerobic degradation of organic matter (Lovley and Chapelle, 1995). The acetate is oxidised in *Geobacter* species using the tricarboxylic acid cycle (Mahadevan et al., 2006) and the genes responsible for acetate metabolism are highly conserved in all the sequenced genomes (Butler et al., 2010).

***Geobacter sulfurreducens*, the model electroactive bacteria**

Geobacter sulfurreducens was isolated for first time in 1994 in Oklahoma from surface sediments from a hydrocarbon-contaminated ditch (Caccavo et al., 1994). The isolated bacteria were initially designated as strain PCA. As a member of *Geobacteraceae* family it is a gram-negative rod and nonfermentative microorganism. Although *G. sulfurreducens* was initially classified as anaerobic (Caccavo et al., 1994) this strain is able to respire oxygen (Lin et al., 2004) under a low concentration of the TEA. *G.sulfurreducens* grows in a defined medium with acetate as an electron donor and iron (III) oxides, iron (III) hydroxides, iron (III)-citrate, elemental sulfur, Co (III)-EDTA, fumarate or malate as the sole electron acceptor. Its ability to couple the oxidation of hydrogen or acetate to the reduction of iron oxides makes strain PCA a unique addition to the relatively small group of respiratory metal-reducing microorganisms available in pure culture. Not only this ability distinguishes PCA but also its capacity to growth in liquid media with fumarate or iron (III)-citrate that facilitates its cultivation as a pure culture in laboratories. Furthermore, it can be grown under continuous culture in a chemostat (Esteve-Nunez et al., 2005), a culture method that allow to achieve a reproducible

physiology key for analysing both transcriptomic (Holmes et al., 2006) and proteomics responses (Ding et al., 2006). In addition, the whole sequenced genome is available (Methé et al., 2003) a genetic system was developed (Coppi et al., 2001) and *in silico* metabolic models were constructed (Butler et al., 2010, 2012; Mahadevan et al., 2006, 2011; Scheibe et al., 2009; Segura et al., 2008).

The role of *Geobacter* as model microorganism for investigating microbial electrochemical systems has been favoured by a) the vast amount of available information about its genes and physiology, and b) the ubiquitous presence of *Geobacter* in microbial communities associated to electrodes.

Extracellular electron transfer in *Geobacter*: the role of C-type cytochromes

The first assays performed to study the iron reductase activity in planktonic cells of *Geobacter* demonstrated that this activity resides in membrane fractions hosting C-type cytochromes (Gaspard et al., 1998; Magnuson, 2000). Later, gene sequences coding for these redox proteins were found in a large abundance and diversity in the analyses of sequenced genomes (Butler et al., 2010; Ding et al., 2006; Methé et al., 2003). *Geobacter* species contain more than 100 sequences for multi-heme C-type cytochromes distributed in 9 families. Surprisingly, the majority of cytochromes are poorly conserved and some families have been found just in one only family (Butler et al., 2010).

During the last decade, efforts were devoted to identify and localize the biomolecules involved in the extracellular electron transport. Genetics and biochemical studies have revealed that

G.sulfurreducens periplasm contains a great number of C-type cytochromes, especially those belonging to the Ppc (periplasmic cytochrome) family (table 1). These proteins have the role of transporting the electrons from the internal transport chain to the outer-membrane (Santos et al., 2015). To date, the electron transfer across the outer membrane towards both iron oxides and electrodes, is proposed to occur mediated by the multi-heme and outer membrane C-type cytochrome OmcB. However, the underlying mechanism has remained uncharacterized. A recent study has reported the existence of an outer membrane porin-cytochrome (Ppc) proteins complex involved in transferring electrons across the outer membrane. This outer complex is composed by a periplasmic C-type cytochrome (OmaB/OmaC), an outer membrane C-type cytochrome (OmcB/OmcC) and a porin-like outer-membrane protein (OmbB/OmbC) (Liu et al., 2014).

In addition, many cytochromes localized outside the cell have also been described. Among them, OmcS (outer membrane C-type cytochrome S), OmcZ and OmcE were found to be important for oxides and electrode reduction (Qian et al., 2011).

Currently, and since the key work of Bulsamen et al. (2008), there have reported a large amount of evidences that not only confirm the presence of C-type cytochromes in the electrical pathway of electron to the TEA but also their role as the last protein that establishes direct contact between bacteria and TEA.

In the table below the most relevant proteins in EET are compiled:

Protein	Local.	Characs.	Likely function	Ref.
PpcA	Periplasm	9,6 kDa. 3 heme groups Constitutive expression	It is able to reduce iron oxides and other metals in vitro. Unlikely contact with insoluble TEA in vivo due to the periplasmic location Translocation e^-/H^+ through inner membrane. PpcA knockout mutant is able to reduce fumarate but not extracellular TEAs such as iron oxides, AQDS and U^{+6} using acetate as electron donor.	(Lloyd et al., 2003)
PpcA homologues: PpcB-PpcE	Periplasm	3 heme groups	Knock-out ppcB, ppcC or ppcE increase the reduction of iron oxides. PpcC polymerizes and has conformational changes in function of redox potential.	(Ding et al., 2008; Morgado et al., 2007; Shelobolina et al., 2007)
OmcB	Embedded in the outer membrane (partially exposed to the extracellular space)	89kDa 12 heme groups	Iron oxides reductase activity. Electric connection between periplasm and extracellular space. OmcB mutant inhibit iron (III)-citrate and iron oxides reduction. Knockout mutants adapt to grow with Fe (III)-citrate but no with iron oxides. OmcB is overexpressed in electroactive biofilms.	(Leang et al., 2003, 2005; Magnuson et al., 2001; Qian et al., 2007; Richter et al., 2009)
OmcS	Outside outer-membrane	47 kDa 6 heme groups	Large abundance of OmcS in iron oxides cultures in comparison with soluble iron cultures. Isolated is able to reduce iron oxides and humic substances. It is also found specifically attached to pilis. OmcS knockout mutant is not able to	(Ding et al., 2008; Leang et al., 2010; Mehta et al., 2005; Qian et al., 2011; Richter et al., 2009)

			grow with iron oxides but it is with iron (III)-citrate. Its expression is reduced in electroactive biofilms.	
OmcE	Outer-membrane surface	32 kDa 4 heme groups	Some kind of role in EET although the knockout mutant was able to revert the phenotype.	(Ding et al., 2008; Holmes et al., 2006; Kim et al., 2008; Mehta et al., 2005; Nevin et al., 2009)
OmpB	Outer-membrane surface	Copper cofactor	It participates in the reduction of iron oxides but not in the reduction of soluble iron-citrate. Co-precipitates with OmcS in co-immune precipitation assays.	(Mehta et al., 2005; Qian et al., 2007)
OmpC	Unknown	OmpB homologue	Important for the iron oxides reduction. It shows different expression pattern from OmpB.	(Holmes et al., 2008)
OmcF	Outer membrane	10kDa 1 heme group	Knockout mutants of OmcF are not able to reduce iron oxides likely due to the absence of OmcB. OmcF could be related to the regulation of OmcB.	(Kim et al., 2005)
Ppc protein complex OmaB/OmaC OmbB/OmbC OmcB/OmcC	Outer membrane	161 kDa 20 heme groups	It participates in the electron transfer across the outer memebrane. Isolated in proteoliposomes demonstrates its ability to transfer electrons across a lipid bilayer.	(Liu et al., 2014)

Table 1. Summary of the main protins involved in EET process in *G. sulfurreducens.*

Although both, the exact role of each cytochrome and the exact pathway of EET remains unknown, there are some elements that are seem to have different roles in the respiration of iron oxides or electrodes. That is the case of the pili structure or nanowire (Malvankar et al., 2011; Reguera et al., 2005). Deletion studies probed that pili are crucial for reduction of iron oxides, but not for other extracellular acceptor such an electrode (Reguera et al., 2005). However, in current producing biofilm growing on electrodes, pili are proposed to provide a conductive network through the internal structure of the biofilm, which allows performing a long-range electron transport (Malvankar and Lovley, 2012).

Three models for long-range electron transport through the inside of *G.sulfurreducens* biofilms have been proposed. For all of them, the proteins and pathways above described are common: electrons transferred through the electron transport chain cross the periplasm and reach the outer membrane following the increasing potentials of the periplasmic cytochrome PpcA. Then, they are transferred to the outer-membrane porin- cytochrome complex. Once the electrons reach this outermost level, these models start to differentiate. Nevertheless, all models include a C-type cytochrome as the last protein in direct contact with the TEA.

-Conduction by pili or nanowires–Metallic-like conduction:

This model is based on the existence of conductive pili that connect OmcB with the iron oxide by performing the electric conduction through the extracellular space. Pili are formed by polymers of mainly one protein, termed pilin or PilA, which is

encoded by the pilA gene. The electron conduction it would take place by resonance due to the packed π-orbitals of aromatic amino acids of the pili subunit (Malvankar and Lovley, 2012; Reguera et al., 2005; Vargas et al., 2013). Interestingly, OmcS cytochrome has been found attached to pili, where it will function as electrical contact to the iron oxide. The model of Metallic-like conduction would mean a new paradigm in the biological electron transfer hence, although filaments are found in a wide diversity of microorganisms, the type IV pili of *G. sulfurreducens* and *G. metallireducens* are the only filaments that have been shown to be required for EET to extracellular electron acceptors or for conduction of electrons through biofilms (Malvankar et al., 2015).

-Conduction by Electron Hopping:

In contrast with the previous model, the EET towards the electrode would occur through the vast network of extracellular cytochromes. Those redox proteins have to be close enough (less than 2 nm) in order to allow the electron transfer from one heme group to a nearby one. In this model, cytochromes as well as other redox molecules (i.e. multi copper proteins) are the responsible of the electron transport meanwhile the pili and extracellular polysaccharides are just support and scaffolding structures (Bonanni et al., 2012b, 2013a; Bond et al., 2012; Robuschi et al., 2013; Schrott et al., 2011, 2014; Snider et al., 2012; Strycharz-Glaven et al., 2011).

-Conduction by stepping stones:

A third model proposes a combined role of both pili and cytochromes in the electron transfer to the electrode. The cytochromes attached to pili would act as nodes in the pili network

connecting aromatic residues of the same filament or adjacent ones. So thus, the cytochromes play the role of intermediaries in the head-tail junction between the monomers that compose the multimeric filament. It is also possible that cytochromes could reduce the energy for the pilin protein reorganization and therefore, accelerates the transfer of electrons (Bonanni et al., 2013b).

Electrochemical set-up and techniques for the analyses of electroactive microoganisms.

The merging of two fields as different as microbiology and electrochemistry have led to integration of specific techniques of each area to elucidate the pathways involved in EET and therefore, to optimize the process.

-Three-Electrode Arrangement:

It is an experimental system that usually includes a potenciostat as the measuring and control device for the system. Three-electrode arrangement consists of:

Working Electrode (WE): an electrode where the reaction of study takes place (i.e. where electroactive bacteria grow respiring it); its potential is controlled versus the reference electrode in a three-electrode system.

Reference Electrode (RE): A non-polarizable (stable) electrode with a fixed potential that sets or measures the potential of the WE.

Counter Electrode (CE): the electrode that allows the reaction at the WE accompanying it with a respective reverse reaction.

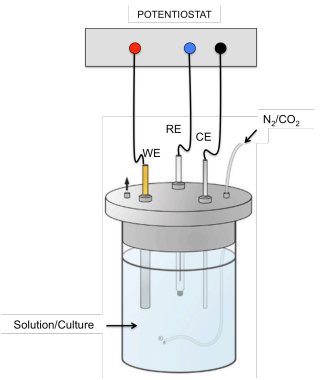


Figure 4. Scheme of the set-up used for the electrochemical analyses of *Geobacter sulfurreducens* cultures.The three-electrode system (WE, CE and RE) was introduced into the reactor under sterile conditions and connected to the potentiostat for the performance of electrochemical measurements.

Partially extracted from (Ma et al., 2013).

-Potentiostat:

An electronic amplifier that controls the potential drop between the WE and the electrolyte solution, it hosts a RE as a sensing component and a CE for balancing the current flow.

-Chronoamperometry (CA):

*T*he measurement of the electrical current production as a function of time is known as a Chronoamperometry.

-Cyclic voltammetry (CV):

This electrochemical analyses consists on the registration of the current production while a potential sweep is

performed along a potential window. It is common to initiate the CV in an initial potential (E_i) from where the sweep can be done towards either positive potentials (anodic sweep, oxidation reactions take place) or negative potentials (cathodic sweep, reduction reactions take place). Once, the extreme potential of the window is reached, the sweep is inverted until reaching the other extreme potential. Depending on the necessities of the assay, the cycle can be repeated *n* times. The speed of the sweep is known as scan rate and is equivalent to the potential variation per unit of time. The scan rate allows obtaining information about the speed of the reactions that occur on the WE.

A CV (Current (I) vs. potential (E)) of a reversible system is shown in Figure 4. In the anodic sweep, no current flow is observed until the potential approaches the standard potential of the redox pair (E^0). Then, the current increases due to an oxidation reaction. As the potential continues to increase, the concentration of the species to be oxidized at the surface of the electrode begins to drop. When the potential reached is higher than the E^0 and the concentration of the specie is almost zero, the current is in its maximum since the mass transport is its highest rate. When the potential increases and the specie is totally consumed, the current eventually drops. The highest current corresponds to a potential known as Peak potential (E_p) and the maximum current is called Peak Current (I_p). When the extreme potential chosen is reached, the sweep is inverted. The shape of the inverse curve (cathodic) depends on the potential at which the sweep was inverted. If the extreme potential chosen is higher than the peak potential, the shape of the inverse curve is the same than the anodic sweep (Bard and Faulkner, 2001).

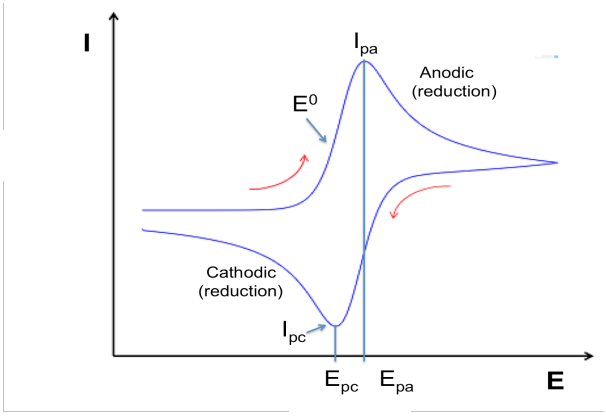


Figure 4. Cyclic voltammetry where E_{pa}: anodic peak potential; E_{pc}: cathodic peak potential; I_{pa}: anodic peak current; I_{pc}: cathodic peak current; E^0: standard potential.

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Chapter 2

Modulating the microbial electroactivity by osmotic shift

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Electroactivity stimulation by bacterial EPS: a key external structure for expanding the biofilm-based paradigm

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ABSTRACT

Microbial Electrochemistry has emerged as a discipline that exploits the direct extracellular electron transfer (DEET) between living cells and electroconductive materials as a fascinating form of respiration. DEET is generally accepted to occur at the electrode interface with a microbial attachment requirement via biofilm, a system where bacteria from the genus *Geobacter* have been consensually considered as model electroactive microorganisms. Interestingly, planktonic cells of *Geobacter* cultured in freshwater medium shows a poor electroactive phenotype. In spite of the biofilm paradigm we have successfully obtained highly electroactive planktonic cells of *Geobacter* after exposing planktonic cells to salt conditions. A proteomic analysis of salt-grown cells showed different strategies for coping with salt stress, especially those related with the synthesis of Extracellular Polymeric Substances (EPS). Moreover, our research has revealed that a poorly electroactive phenotype can shift to highly electroactive just by adding EPS isolated from salt-grown *Geobacter*. The salt-grown EPS was interrogated through Z potential measurements, nanoparticle-enhanced Raman spectroscopy (NERS) and proteome composition, and the analysis revealed that OmcS, a C-type cytochrome previously reported as key for electricity production, was up regulated.

INTRODUCTION

Geobacter sulfurreducens belongs to the group of metal-reducing microorganisms, well known by its ability to couple the oxidation of acetate or hydrogen to the reduction of insoluble oxides located outside the cell (Caccavo et al., 1994) in a process known as Direct Extracellular Electron Transfer (DEET). The phenomenon of DEET makes *G.sulfurreducens* to have an essential role not only in ecology and biogeochemistry of soils and sediments (e.g. iron cycle) (Lovley et al., 1987, 2004) but also in the emergent platform of Microbial Electrochemical Technologies (MET) (Schröder et al., 2015). In METs, *G.sulfurreducens* is the model organism for transferring electrons from organic matter oxidation to a conductive material (electrode) that behaves as extracellular electron acceptor (Bond and Lovley, 2003). Therefore, DEET becomes the core reaction in a number of applications focused on energy harvesting from waste (Microbial Fuel Cell, MFC)(Rabaey and Verstraete, 2005), desalinating water (Microbial Desalination Cell, MDC) (Cao et al., 2009), microbial electrosynthesis (Rabaey and Rozendal, 2010) and even in biorremediating polluted environments (Microbial Electroremediating Cell, MERC) (Rodrigo et al., 2014; Rodrigo et al., 2016), among others.

The process of DEET is viable in *G.sulfurreducens* due to the vast network of C-type cytochromes coded by up to 100 genes in its genome (Methé et al., 2003), where most of them host multiple heme groups that act as electron carriers. These redox proteins are the ones which establish direct physical contact between the internal cytoplasm and the extracellular environment (Aklujkar et al., 2013). Their role in the electrochemical communication with the electrode have been demonstrated in several studies (Busalmen et al., 2008; Esteve-Núñez et al., 2011; Strycharz-Glaven et al., 2011) . Moreover, either a severe reduction of the cytochromes C content (Estevez-Canales et al., 2015) or the knockout of specific cytochromes (Mehta et al., 2005; Orellana et al., 2013) can trigger a DEET failure.

Furthermore, *G.sulfurreducens* can grow in a biofilm structure where cells in layers up to 100 μm from the electrode are still able to respire it through DEET process. From cell cytoplasm to the outermost-membrane, the electrons must cross the periplasm using strictly a cytochrome-based process. Once the electrons reach the outermost-membrane, there are several electron transfer models to explain the electron way towards the electrode through different key redox structures inside the biofilm matrix. A proposed mechanism suggests pili as the exclusive extracellular and proteinaceous structure involved in the conductivity across the biofilm (Metallic-Like Conduction Model) (Malvankar and Lovley, 2014; Reguera et al., 2005; Vargas et al., 2013), although it is a cytochrome the last player in contacting with the electrode. On the other hand, the Electron Hopping mechanism proposes a conductivity process just among nearby cytochromes (Schrott et al., 2011; Snider et al., 2012; Strycharz-Glaven et al., 2011), while the Stepping Stones involves a cytochrome-pili combination (Bonanni et al., 2013).

In spite of being DEET an outermost cell process in *G.sulfurreducens*, the biofilm matrix or the extracellular polymeric substances (EPS) have been hardly explored in depth further as a structural piece. Roffelson et al. (2011) (Rollefson et al., 2011) was one of the pioneers in showing the importance of this extracellular matrix for the formation of *Geobacter* biofilms as anchoring structures for the attachment of C-type cytochromes. Some more recent studies (Strycharz-Glaven et al., 2014) have considered the EPS more than just an scaffolding structure, but as an electrically conductive link that can narrow the long-distance for transferring electrons outside the cell.

In order to boost METs to a real technological option, highly electroactive biofilms are desired (i.e. higher current generation). To this end, either genetic engineering (Leang et al., 2013; Wang et al., 2013) or adaptation approaches based on the electrode potential have been reported (Yi et al., 2009). Furthermore, alternative strategies based on increasing the conductivity of the anodic medium (i.e. improving current output by decreasing internal resistance)

have revealed an enhancement of the *Geobacter* electroactive capacity (Miyahara et al., 2015) or even lead to the isolation of hypersaline *Geobacter* strains (Sun et al., 2014).

Biofilm is the grown mode where *Geobacter* has been consensually considered as the electroactive model bacteria, and, consequently, the biofilm-based physiology as the paradigm from electricity-harvesting bacteria. However, this bacterial genus have performed DEET for millions of years in groundwater, their natural habitat (Childers et al., 2002; Holmes et al., 2002), in a cellular planktonic state. Interestingly, planktonic cells of *Geobacter* cultured in freshwater medium shows a poor electroactive phenotype. With the aim to expand the electroactive capacity from biofilm to planktonic cells a number of reports have explored *Geobacter´s* physiology (Borjas et al., 2015; Esteve-Núñez et al., 2011).

In this work, we present evidences that support how a salt-supplemented growing media was able to enhance the electroactivity of *G.sulfurreducens* by 3-fold. This highly electroactive phenotype is associated to the subsequent osmotic response, a shift in the EPS. In addition, we confirm the EPS role in DEET and propose it as an electroactive network to plug planktonic *G.sulfurreducens* cells into electrodes suggesting novel applications where a controlled electroactive response is required.

MATERIALS AND METHODS

In order to study the electron transfer of *Geobacter sulfurreducens* to electrodes under salt conditions, glass reactor were used for the microorganism culture where electrochemical measurements were taken. Reactors were inoculated with pure batch culture of *G.sulfurreducens*. For all the assays, fresh water medium (described below) was used as standard medium (FWM-0) and artificially supplemented with different sodium chloride concentrations with 5 $g.l^{-1}$ NaCl (FWM-5), 10 $g.l^{-1}$ NaCl (FWM-10) or 30 $g.l^{-1}$ NaCl (FWM-30).

Bacterial strain, culture conditions

Bacterial strain used along this research was DL1 of *Geobacter sulfurreducens.* It was routinely grown at 30 ºC in several Pyrex bottles containing Freshwater medium (FWM) (pH 6.9, EC 12,4 mS/cm) with the following mineral salts: $NaHCO_3$ 2.5 g l^{-1}; NH_4Cl 0.25 g l^{-1}; $NaH_2PO_4H_2O$ 0.06 g l^{-1}; KCl 0.1 g l^{-1}; $Fe(NH_4)_2(SO_4)_2\ 6H_2O$ 0.04 g l^{-1}, and was supplemented with a trace minerals and vitamins solutions. Sodium acetate (20 mM) was used as electron donor and fumarate (40 mM) as sole electron acceptor as described elsewhere (Esteve-Nunez et al., 2005). The corresponding NaCl amount was added. The culture media were anaerobic due to the gassed of a mixture of N_2/CO_2 (80:20, industrial ALIGAL-12). Passing through heated copper fillings gas mix was deoxygenated. In order to obtain the growth curves of the microorganism in different conditions, the turbidity of cultures were measured every 24 h by spectrophotometry (600nm, UV-1800, Shimadzu UV spectrophotometer). In the case of cultures with cell aggregates, samples were sheared by five passes through an 18-gauge needle. For electrochemical measurements, bioreactors with a 200 ml volume containing or FWM-0 or NaCl supplemented media (FWM-5 or FWM-10) were inoculated with exponential phase *Geobacter sulfurreducens* batch culture in a rate 1:10. A constant temperature of 30 ºC was kept through the bioreactor with an outer water jacket that was connected to a water bath with recirculation (Isoterm 3016H, Fisher Scientific). Each bioreactor had a constant stirring with magnetic

bar. Anaerobic atmosphere was kept with a constant flow of N_2/CO_2 (80:20, industrial ALIGAL-12).

Electrochemical assays

For the electrochemical characterization by voltamperommetry and chronoamperometry, a three-electrode system was used. It was composed of a working electrode and a counter electrode both consisted in graphite rods (Mersen S.L.) with a 0.4 cm diameter and 10 cm length that were partially submerged (3.7 cm^2 area in contact with culture). The system was completed with a reference electrode (Ag/AgCl 3.5 M de KCl, BASi Reference Electrodes) located next to working electrode. Graphite rods were first washed with HCl 0.1 mM and sanded down (grain 180). All connections were done with Au wire in order to avoid interferences by corrosion. In each assay the following measurement sequence was followed: 1) Working polarization at 0.2 V vs. reference electrode and chronoamperometry during 20 min. Then, cyclic voltammetry at 5 $mV.s^{-1}$ was performed (between -0.8 and 0.8 V). For the polarization, a potenciostat was used (PGZ100, Voltalab Radiometer Analytical). All experiments were done at a controlled temperature of 30 ºC and under anaerobic conditions. It is important to mention that all assays were performed at short terms periods where no electroactive biofilm could be formed. The aim was to test electroactivity in planktonic cells.

The same three-electrode system was used in order to test the electroactivity of cultures with EPS extracts addition.

Scanning Electron Microscopy (SEM)

Planktonic cells from each reactor were washed with bicarbonate buffer and harvested by centrifugation (8000 x *g*, 10 min). Cell pellets were recovered and fixed by immersion in 5% in 0,2 M Cacodylate buffer and dehydrated by a graded ethanol series (25, 50, 70, 90 and 100 %). Finally, dehydrate cells were CO_2-critical point dried and processed using an scanning electron microscope

DSM-950 (Zeiss) by the microscopy service of Alcalá University.

Zeta Potential measurement

There is not a method for directly determining surface charge, but it has been proven that an indirect determination of zeta potential by the technique of electrophoretic light scattering (ELS) is an useful alternative (Wilson et al., 2001). Therefore, A Zeta potential equipment (ZetaSizer Nano-ZS, Malvern Instruments) was used to determine Zeta potential values of each culture. Samples were obtained by suspending 3.10^8 cells of *G.sulfurreducens* cells grown in FWM-0, in FWM-5 and in FWM-10 medium, in 30 mM of sodium bicarbonate buffer at pH 7.0. Five measurements of each culture were taken.

Extracellular polymeric substances (EPS) isolation

In order to isolate the extracellular matrix attached to cells cultivated in FWM-0 and in FWM-5, a procedure previously described for *Geobacter sulfurreducens* (Rollefson et al., 2011) was used. Cells were harvested by centrifugation (8000 x *g*, 10 min) and suspended in TNE buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM EDTA) and blended in a commercial blender. Cells were treated with SDS (0.1 %) for their lysate. Several rounds of centrifugation (17000 x *g*, 15 min) followed by Tris-HCl (pH 7.5) washes for the removal of SDS were performed.

Analysis of isolated EPS

The phenol sulphuric acid method was used to detect carbohydrates with glucose as standard (Masuko et al., 2005). For the detection of total proteins, Bradford method with BSA (bovine seroalbumine) as standard was used.

Protein identification of *Geobacter sulfurreducens* cells

In order to obtain whole-cell proteome of FWM-0 and FWM-5 media grown cells, cellular protein solubilisation was performed. The same number of

cells from both conditions were harvested by centrifugation (8000xg, 10 min) and washed with 1X PBS buffer. Cells were lysed by ice-incubation with lysozyme (0.2 µg/ul) for 30 min. Then, proteins were solubilized and reduced with the addition of 1% of Triton X-100 and 0.05% β-mercaptoethanol followed by a sonication treatment of 15 min. Proteins in this cell lysate were denatured by the addition of urea (8 M). Finally, pool proteins were passed by 0.8 mm and 0.6 mm gauge needle in order to avoid the existence of any aggregates. Final protein extracts were obtained in the supernatant of the final 12000 x *g* centrifugation of 5 min. It was preserved at -20ºC until its analysis. The protein identification was carried out in the CBMSO PROTEIN CHEMISTRY FACILITY that belongs to ProteoRed, PRB2-ISCIII and where samples were desalted and concentrated by Zip-Tip/OMIX. Protein identification was performed by high resolution- medium gradient LC-MS/MS. Results were obtained by SEQUEST software with *Geobacter*-Uniprot database.

EPS incubation assays

FWM-0 was prepared as describe above and then supplemented with a volume of 10% (v/v) of anaerobic EPS extract. Once it was added, regular inoculation with *G.sulfurreducens* was performed. Electrochemical measurements were taken when stationary-phase was reached.

Nanoparticle-enhanced Raman spectroscopy (NERS)

NERS measurements were performed using a LabRAM HR800 confocal Raman microscope (Horiba JobinYvon). The excitation wavelength was 532 nm (Nd:YAG laser). The power of the laser on the sample was 1 mW, and a 50 times magnification long-working-distance objective (8 mm) with a numerical aperture of 0.1 was used to focus the laser onto the sample to collect the scattered light in a backscattering geometry.

Citrate-stabilized Ag nanoparticles (NPs) with a mean diameter of 80 nm were synthesized by reducing 200 mL of a boiling 0.018 wt% $AgNO_3$ solution with 4 mL

of 1 wt% sodium citrate. A colour change from yellow to pale green took place within 20 s. The solution was kept boiling for 4 h, and was then stirred until cooling down to room temperature. 1.5 mL of the NP solution was subsequently cleaned and concentrated by a factor of 100 via centrifugation (5500 rpm for 15 min, 3 times), which led to 10–20 μL of a dark green sediment. This sediment was mixed with concentrated *Geobacter*-EPS sample (5000 rpm for 4 min, 3 times) and subsequently cast and dried on a glass slide in an Ar atmosphere.

RESULTS

Ion conductivity of culture media and microbial electrochemistry are intimately related due to the requirement of an efficient ions migration towards electrodes for an optimal METs performance (Hamann et al., 2007). In METs, a slow rate of ions transfer is limiting step for power generation that can be overcome by increasing the salinity of the system (Fan 2008; Gil 2003). A higher salt concentration, either in the cathode or the anode electrolyte, could lead to a salt stress imposition to the electroactive biofilm (Heilmann and Logan, 2006; Lefebvre et al., 2012; Liu et al., 2005; Miyahara et al., 2015). Based on the benefits of using more conductive electrolytes in METs device (Nam and Logan, 2011; Wang et al., 2011) we explored the salt tolerance of the electroactive model bacteria *Geobacter sulfurreducens.* In contrast with the paradigm of studying electroactive cells as a biofilm, in the current work we aimed to explore the electroactive physiology of planktonic cells. So thus, *G.sulfurreducens* planktonic cells were cultured in standard freshwater medium (FWM-0) supplemented with increasing concentrations of NaCl (5,10 and 30 $g.l^{-1}$). Results demonstrated that *G.sulfurreducens* was able to growth under salt conditions, reaching optical densities even higher than those in standard freshwater conditions (Figure 1S). Nevertheless cell aggregation was observed at NaCl levels as high as 10 $g.l^{-1}$, and no growth at all was observed at 30 $g.l^{-1}$ of NaCl.

Salt-grown planktonic *Geobacter* cells are highly electroactive

With the objective of testing the electroactivity of planktonic cells, a series of short-term assays under biofilm-free conditions were conducted. Electrochemical analysis of FWM-0, FWM-5 and FWM-10 cultures was performed revealed a current density 3-fold higher in cells from FWM-5 medium. Even more remarkable was the cyclic voltammetric response of salt-grown cells, showing a redox couple with oxidation process at 0 mV and a reduction process at -300mV. These peak where also observed in the FWM-0-grown cells, but the intensity was

several orders of magnitude smaller. (Figure 1A and 1B). The higher peak current (I_p) displayed by salt-grown cells indicates a higher amount of redox molecules in electrical contact to the electrode. On the other hand, the addition of NaCl to the freshwater medium increased the electrical conductivity (CE) of the growth culture from 12.4 mS/cm to 19.84 mS/cm (for 5 $g.l^{-1}$) and 27.5 mS/cm (for 10 $g.l^{-1}$). In order to evaluate the influence of conductivity on the electrochemical performance of bacteria cells, FWM-0-grown cells were supplemented with NaCl (5 $g.l^{-1}$) just before performing the electrochemical analysis. Interestingly, the higher conductivity was not directly causing any enhancement in the electroactivity of freshwater-grown cells according to CV and chronoamperometric analysis. This result pointed towards growth-associated physiological changes as the reason for such an electroactive phenotype. Notably, cells grown with FWM-10 showed a lower I_p than the ones grown with FWM-5.

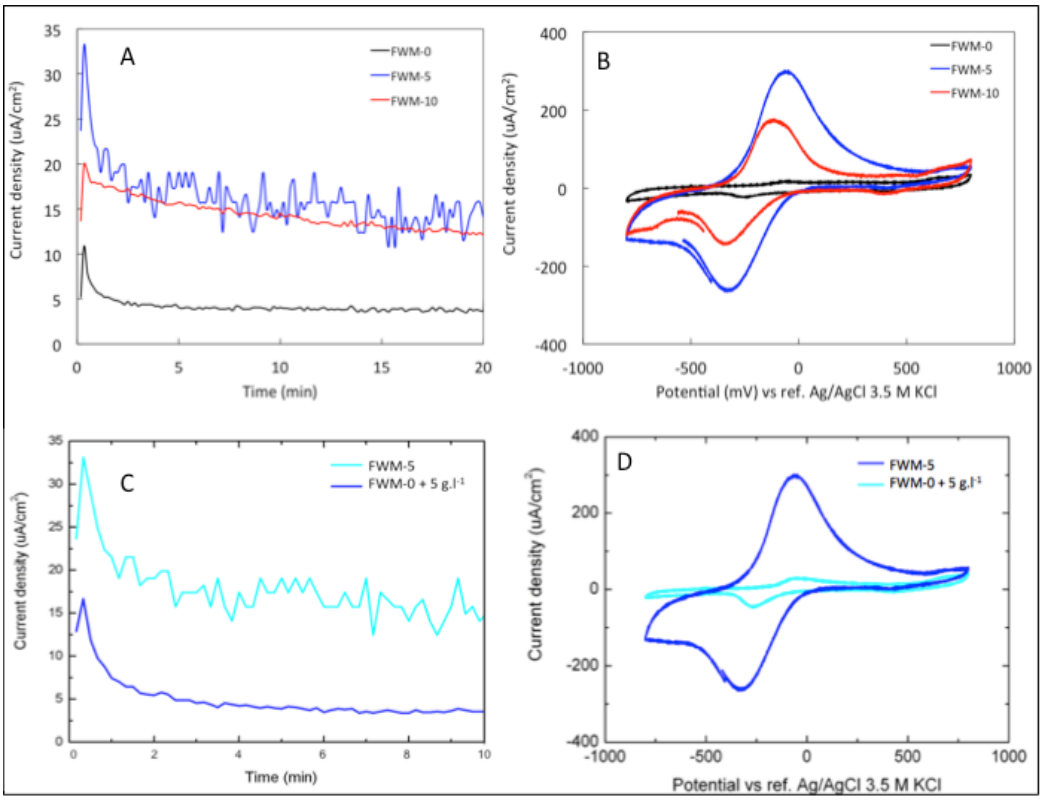

Figure 1. (A) and (B) are chronoamperommetric analysis and cyclic voltammetric analysis respectively of *Geobacter sulfurreducens* grown in FWM-0 (black line), in FMW-5 (blue line) and in FWM-10 media.(red line)frewshwater medium). (C) and (D) are chronoamperommetric analysis and cyclic voltammetric analysis of *Geobacter sulfurreducens* grown in FWM-5 medium (dark blue line) and grown in FWM-0 medium supplemented with 5 g.l^{-1} NaCl just before the electrochemical assay (light blue line), respectively.

Interrogating the proteome of salt-grown electroactive *Geobacter*

Considering the ability of *G.sulfurreduncens* to grow under salt conditions, the development of an osmotic response might be implicated. Furthermore, the different bioelectrochemical response of salt-grown cells suggests a significant physiological change that should be correlated with variations in the proteome. Hence, in order to go in depth in this stress adaptation, cells cultured either in

FWM-0 medium or in presence of additional NaCl, FWM-5 medium, were interrogated regarding their proteomic profile.

In the analysis, a total number of 3396 proteins were detected which represent ~98% of the 3469 total proteins predicted in the genome of *Geobacter sulfurreducens* (Methé et al., 2003). In salt-grown cells, 178 proteins showed a higher abundance compared with the control cells grown in FWM-0, in contrast with the 304 proteins shown in lower abundance. This number of significantly altered proteins, represent a 14.2 % of the total proteins detected, indicating that protein synthesis was significantly affected by the presence of NaCl in the media.

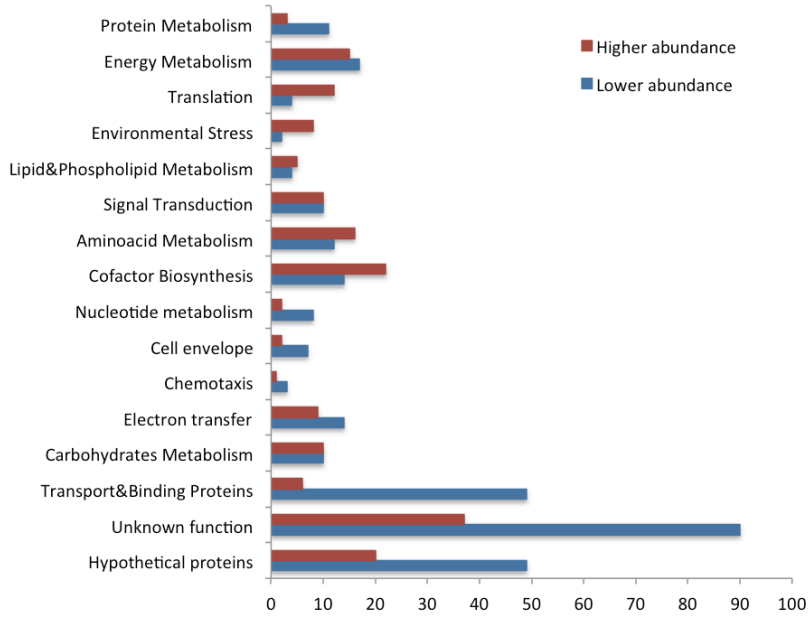

Figure 2. Changes in the protein profile after NaCl exposure. Blue bars are the number of proteins with a lower abundance. Red bars, proteins with an increased relative abundance. Categories were assigned based on the function annotated in *Geobacter sulfurreducens* genome.

The proteins that showed a different synthesis profile were classified under 15 categories according to their annotation function in the genome (Figure 2). Proteins associated with environmental stress were in a higher number in presence of NaCl, e.g. some heat shock proteins (HSP) which synthesis is induced

by osmotic shock in several microorganisms (Kilstrup et al., 1997; Sherman, 1987). Other proteins involved in amino acid and lipid metabolism were found in a higher presence too, as well as a large number of proteins classified as hypothetical proteins. Most of the proteins showing a lower abundance after NaCl exposition was annotated as transport and binding proteins. The rest of the proteins in lower abundance belong to the other categories showing such as nucleotide metabolism and motility.

Proteins involved in the osmotic response of the electroactive planktonic *Geobacter*

The addition of NaCl to the growth media increases the extracellular osmotic pressure, what entails a rapid water efflux that concentrate the cytoplasm of living cells disrupting their structure and function. In order to avoid this, cells respond to osmotic stress by actively adjusting the distribution of solutes across the membrane. This adjustment takes place mainly by two mechanisms (Oren, 2008). The first one is the "salt-in" strategy, which involves accumulation of potassium. K^+ ions are the most prevalent cations in the bacteria cytoplasm (Shabala et al., 2009) and therefore, one of the main intracellular osmolytes that maintain turgor. Interestingly, the high-affinity ATP-driven potassium transport system (kdpB, GSU2481) was more abundant in *G.sulfurreducens* cells exposed to NaCl, suggesting a higher exchange of hydrogen and K^+.

The second strategy (Oren, 2008) consists in the accumulation of organic solutes (osmolytes) either by synthesis or by transport from the extracellular medium without impairing cell functions. Indeed, in *G. sulfurreducens* cells exposed to NaCl, a higher abundance of proteins involved in the biosynthesis of amino acids, such as aspartate and glutamate (nfnA, GSU3057) as well as glycine, serine and threonine (ltaA, GSU 3162) and histidine (hisF, GSU3095 and hisH, GSU3097) were detected.

The *G. sulfurreducens* proteome analysis also revealed how the exposition

to NaCl triggered the production of the protein N-carbamylputrescine amydase (aguB, GSU1027) which is involved in putrescine synthesis, a divalent cation, whose rapid excretion can increase the strength of cytoplasm and balance the accumulation of K^+ (Janet M. Wood, 1999; Schiller et al., 2000)

Trehalose is one of the important compatible solutes that bacteria produce in response to an osmotic stress (Csonka, 1989; Janet M. Wood, 1999). Interestingly, Trehalose-6-phosphate synthase (otsA, GSU2337) and maltooligosyltrehalose trehalohydrolase (treZ, GSU2358) were also found at higher abundance in *Geobacter* after growing on NaCl.

Regarding the oxidative metabolism, proteins of salt-grown *G.sulfurreducens* cells displayed no significant changes. For instance, the presence of tricarboxilic acid (TCA) cycle proteins was slightly reduced and a protein as the bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase (ancB, GSU1660) as well as succinyl-CoA synthetase (GSU1059), kept the same abundance level regardless the presence of salt. Furthermore, the process of protein biosynthesis was actually triggered by the presence of NaCl. This was observed as a higher abundance of proteins involved in translation such as the elongation factor Ts (GSU1920), elongation factors ABCtransporter (GSU0922) as well as several ribosomal proteins, such as RplA, RpsA, RplF, RpmG, RplL, RplO, RplR, RplV.

Among the protein categories affected by the NaCl upshift in *G.sulfurreducens* cells, those related to transport and binding proteins were the greatest altered. For instance, 3 different types of transport systems were in a lower abundance: ATP-binding cassette (ABC) system for the transport of amino acids (GSU0799), metal ions (GSU1332) and carbohydrates (GSU1889) (Roth et al., 1985). Moreover, facilitated diffusion transport for small solutes included oligosaccharides, metabolites, etc. (GSU1480) were also downregulated.

The inhibition of the main routes of transport in the cell, except the ones for osmoregulation as the K^+ influx pump, can be attributed to conformational changes of the different carriers (Houssin et al., 1991) caused by the deformation

of the membrane subjected to the high-osmotic pressure. This deformation in the curvature that undergone the membrane exposed to high-osmolarity media, reveal an important role in the osmoregulatory process. Actually, mechanosensitive channels involved in osmoregulation are opened in response to forces exerted by the lipid layer (Teng et al., 2015) due to the membrane deformation. In the case of *G.sulfurreducens*, the low abundance of proteins as lptA (GSU1889), was an indicative of an outer membrane defect (Tran et al., 2008). Meanwhile this kind of response is activated by membrane deformation, bacteria can control and adjust their membrane composition by modifying the types of fatty acids that are produced by biosynthetic pathway and altering the structures of pre-existing phospholipids, both mechanisms essential for the bilayer stability in cells (Zhang and Rock, 2008).

Interestingly, the proteins related to fatty acids and phospholipid metabolism in *G.sulfurreducens* were found in a higher abundance after the exposure to salt conditions. This was the case for SGNH-hydrolase lipoprotein (GSU2415) involved in the biosynthesis of unsaturated fatty acid (characteristics in extracellular matrixes), Acyl-glycerolphosphate (GSU2072) related to lipid biosynthesis proteins. Moreover, lipid-A-disaccharide synthase (lpxB, GSU2261), phosphoheptose isomerase (gmhA, GSU2087), UDP-N-acetylglucosamine acyltransferase (lpxA, GSU0999), all of them precursors of lipopolysaccharide synthesis, were found in higher abundance too.

In order to cope with the stress imposed by salinity, biofilm formation and exopolysaccharide (EPS) production are well known strategies of salt tolerant bacteria to assist metabolism and survival (Qurashi and Sabri, 2012). Apparently, the development of biofilm is linked to the signalling molecule c-di-GMP (3´,5´-cyclic diguanylic acid) (Cotter and Stibitz, 2007). The enzyme that is responsible for the synthesis and degradation of c-di-GMP is the GGDEF (diguanylate cyclase), and its expression is controlled by an environmentally responsive signal transduction systems. Futhermore, it has been reported that the biofilm matrix can play an active role in stimulating its own synthesis (Irie et al., 2012). In the

case of *G.sulfurreducens* cell exposed to NaCl, the synthesis of DCG (Diguanylate cyclase, GSU0474) was higher, likely indicating larger amount of the second messenger c-di-GMP and therefore a possible higher tendency to EPS synthesis.

Proteins involved in electroactive response of *Geobacter*

Regarding In relation to electron transfer mechanism, three main types of proteins were analysed: 1) C-type cytochromes, in contrast to what was expected from cyclic voltammetry results, the cytochromes identified in NaCl-supplemented cultures were in a slightly lower abundance than in freshwater sample; 2) electron transfer flavoproteins, several subunits of these electron shuttles were in higher abundance (GSU2797, GSU2796) after NaCl exposure; 3) pili proteins, proteins related to the synthesis and assembly of pili were not altered or even in were detected in a lower abundance respect to the standard FWM-0-grown cells.

SEM and Zeta potential: cell surface changes under salt conditions

Deeper studies on the cell surface should be carried out considering the tendency of salt-grown *Geobacter* cells to form aggregates as well as the changes in the machinery for constructing the outermost region of the cell revealed by the proteome analyses, The synthesis of EPS suggested by the proteome (DGC synthesis) were lately confirmed by SEM analyses. In Figure 3, SEM analyses make evident the presence of an extracellular structure around bacteria in NaCl-supplemented media.

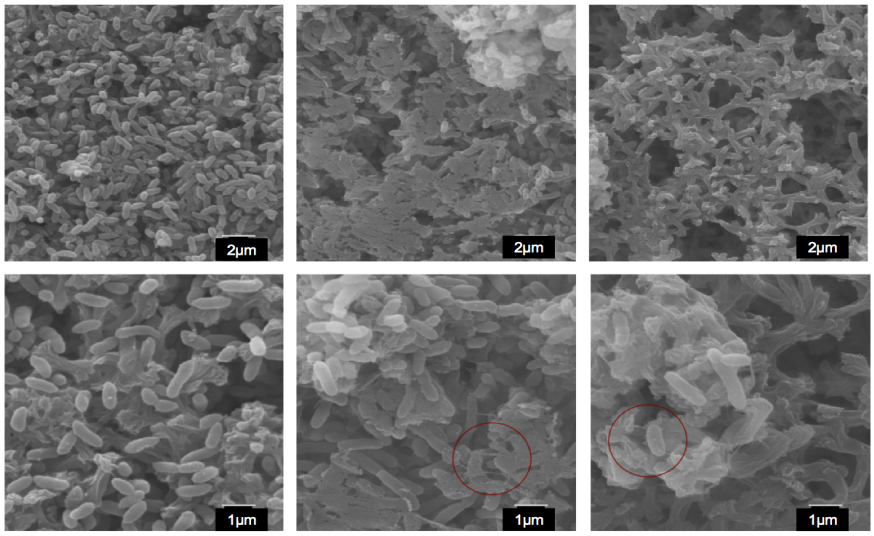

Figure 3. SEM pictures from *Geobacter sulfurreducens* batch cultures in different salt conditions (FWM-0 (left), FWM- 5 (middle) and FWM-10 (right) of NaCl. Red circles show material interconnecting cells.

Further confirmation comes from zeta potential measurements. This is the electrical potential of the interfacial region between the bacterial surface and the aqueous environment (Saito et al., 1997). So, changes of the outermost region like the EPS presence would affect the values of Z potential. Actually, *G. sulfurreducens* cell surface electronegativity increased when NaCl concentration was raised (Table S1). The negative values of the zeta potential were probably due to a mayor presence of acidic functional groups (e.g. carboxyl, phosphoryl or sulfhydryl groups of carbohydrates and proteins) within the EPS matrix on the cell surface. In addition, it has been reported that microorganisms hosting a large EPS exhibit more electrophoretic mobility and consequently a more negative zeta potential (Planchon et al., 2013; Postma et al., 1991). In order to verify this, EPS matrix from both FWM-0-grown and FWM-5-grown *Geobacter* were studied.

Sample/ NaCl Condition	Proteins (mg/ml)	Carbohydrates (mg/ml)
FWM-0	0.054	0.047
FWM-5	0.087	0.073
FWM-10	0.11	0.123

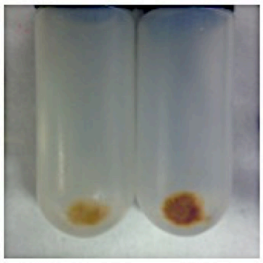

Table 1. EPS-associated proteins and carbohydrates content (mg/ml) from *G.sulfurreducens* cultivated in FWM supplied with 0, 5 $g.l^{-1}$ and 10 $g.l^{-1}$ NaCl.

Figure xx. EPS extracts from *G.sulfurreducens* cultured in FWM supplied with 0 $g.l^{-1}$ NaCl (left) and 5$g.l^{-1}$ NaCl (right).

Based on the carbohydrates sensibility to hard acids and high temperatures, Dubois method showed that salt-grown *Geobacter* shows 3-fold higher abundance of carbohydrates than the FWM-0-grown cells. Moreover, the abundance of proteins was almost doubled under salt-conditions (Table 1). Our analysis revealed a negative correlation of Zeta potential with the hydrocarbon and protein content in EPS. (Figure S2)

Expanding the electroactive phenotype by addition of an EPS matrix

Our previous results revealed that a poor electroactive phenotype can shift to a highly electroactive just by growing the cells on a salt medium as high as 5-10 $g.l^{-1}$ NaCl. Indeed, the shift of the EPS might be responsible of such an enhanced-electroactive phenotype. In order to test such an EPS-based hypothesis and give insights into the role of EPS from salt-grown cells, we proceed to perform the following *in vivo* assay. FWM-0-grown planktonic *Geobacter* cells were incubated with EPS (10%) isolated from salt-grown cells. The incubation assay did not revealed any toxic effect since both wild type and EPS-supplemented cells where showing the same generation time (ca. 25 hours). EPS-supplemented cells led to a novel phenotype showing a high electroactive response according with cyclic voltammetry analysis (Figure 4) where enlarger redox peaks were observed. The oxidative peak remains the same that under salt-grown cells, although the reduction potential showed some shift to more negative potential. This shift might be related to the interaction bacteria-electrode since it has been previously reported that, even using the same electrode material, a mere change in the active surface could entailed a different microorganism electroactive behaviour (Maestro et al., 2014). Interestingly, isolated EPS from salt-grown cells did not show any *in vitro* electroactivity against a graphite electrode (Figure 4), suggesting that the *in vivo* interaction of the EPS with living cells are the cause for all redox peaks detected. Moreover, that EPS-FWM5-grown cells showed a 3-fold enhancement peaks in the cyclic voltammetry analysis, revealing that cells are active in coupling oxidation and extracellular electron transfer. Somehow the EPS isolated from salt-grown-EPS was able to enhance the connectivity between *Geobacter* outermost surface and the electrode.

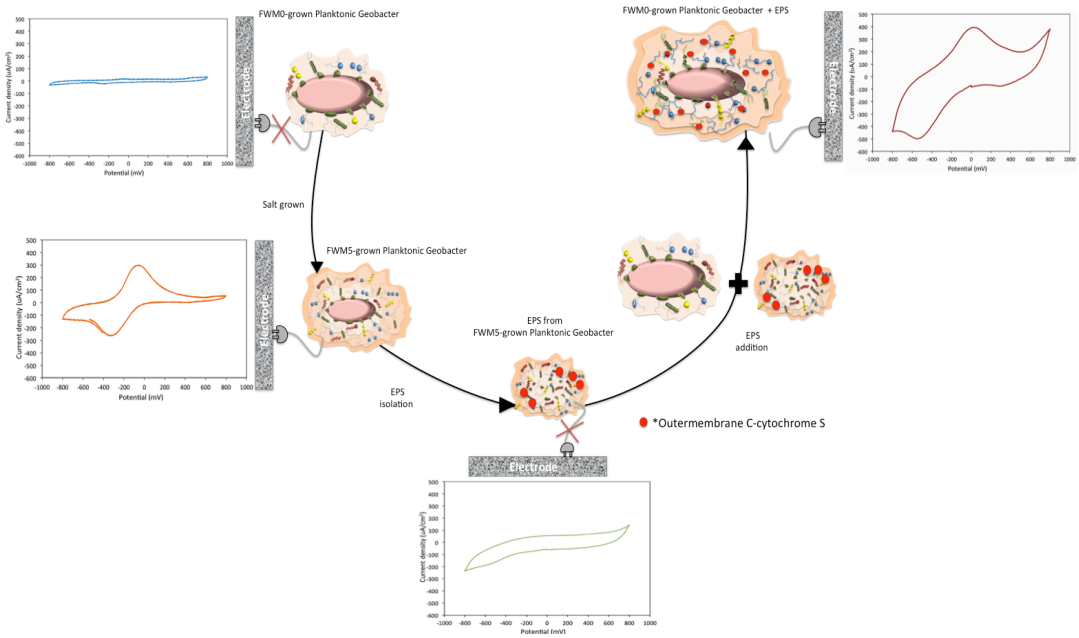

Figure 4. Cyclic voltammetry analyses of a) FWM-0 planktonic cells; b) FWM-5 planktonic cells; c) isolated EPS from FWM-5 cells; and d) FWM-0 planktonic cells incubated with EPS from FWM-5 cells. The scheme illustrates how the addition of EPS from FWM-5-grown electroactive *Geobacter* cells can convert non-electroactive planktonic cells into electroactive ones.

The cyclic voltammetry analysis for both salt-grown cells and EPS-supplemented FWM-grown cells revealed a large increase in the peak current. The fact that this response is due to the EPS matrix suggests that the matrix can either provide with more redox species which connect cells with the electrode, or another kind of electric attachment (no necessarily cytochromes one) that improve the already connection cells-electrode.

Interrogating the EPS composition from salt-grown *Geobacter* cells

Salt stress by itself has been reported to trigger the synthesis of extracellular polymeric substance (EPS) and ultimately to facilitate the biofilm formation (Flemming and Wingender, 2010). Although EPS were previously

thought to be mainly composed of a mix polysaccharides (Eboigbodin and Biggs, 2008), nevertheless further studies have demonstrated that there are also involved proteins, lipids, humic acids and even nucleic acids (Allesen-Holm et al., 2006).

In order to give insights into the electron transfer role of the EPS we decided to perform a proteomic analysis of EPS isolated from both FWM0-grown and FWM5-grown *G. sulfurreducens* cells.

The proteomic analysis of EPS extracts from salt-grown *Geobacter* cells revealed a total number of 1216 proteins that represent ~35% of the 3469 total proteins predicted in the genome of *Geobacter sulfurreducens* (Methé et al., 2003). Interestingly, cyclic AMP receptor protein (cAMP), a cytoplasmic protein used as an indicator of contamination by intra- cellular materials (Cao et al., 2011), was not identified in our EPS extracts suggesting that the EPS isolation protocol was properly performed. In this assay 402 proteins, out of the 1216 identified, were showing a higher abundance in EPS from salt-grown cells compared with the EPS from freshwater-grown cells. Interestingly, 145 proteins were showing a lower abundance. The number of altered proteins represents a 45 % of the total proteins detected, indicating that protein expression in EPS was highly affected by the presence of NaCl in the media. The proteins that showed a different abundance profile were classified under 13 categories according to their annotation function in the genome and represented in Figure S3.

Since *Geobacter´s* cytochromes C were reported to be responsible for transferring electrons *in vivo* to a gold electrode (Busalmen et al., 2008), several techniques involving infrared (Busalmen et al., 2010; Esteve-Núñez et al., 2011) and Raman spectroelecrochemical analysis (Kuzume et al., 2013; Millo et al., 2011) have been used for exploring bacterial surface. Indeed, With the aim to analyse changes in the matrix of EPS, a nanoparticle-enhanced Raman spectroscopy (NERS) was performed and Figure 5 displays a NERS spectra of EPS extracts mixed with Ag nanoparticles and cast-dry on a glass surface where we observed heme-related bands (ν10, ν2, ν11, ν3, ν20, ν4, ν21, ν13, ν30, ν22 and

ν15). This result demonstrates the presence of heme groups in both samples (EPS extracts from FWM0 and FWM5-grown *Geobacter* cultures). The key heme-related bands of NERS spectra are assigned in Table S2.

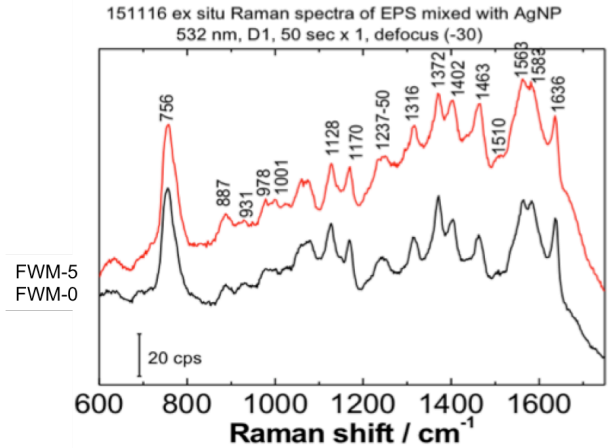

Figure 5. Silver-Nanoparticle-enhanced Raman spectra of EPS from cells cultured in FWM-0 and in FWM-5 medium.

The proteome results confirmed also the presence of cytochromes, which were detected in a moderate abundance with two interesting exceptions that were triggered: a group of flavoproteins and the outer membrane C-type cytochrome, OmcS. The last one has been reported to have an important role for both the DEET to iron oxides and electrodes under a number of conditions (Holmes et al., 2006; Mehta et al., 2005). The presence of a higher abundance of this cytochrome could explain the results obtained in the EPS transfer assay since, the localization of OmcS in alignment with the proteinaceous nanowire structure, pili (Leang et al., 2010) suggested a role in electron transfer intrabiofilm through biofilm matrix (Bonanni et al., 2012, 2013) . In our system, in spite of a higher abundance of OmcS, no proteins related to synthesis or assembly of pili were identified in EPS extracts.

CONCLUSIONS

Microbial electrochemical systems are suitable technologies for coupling metabolism with extracellular electron transfer to electrodes. However, a number of factors should be explored in order to optimize the methodology and make them feasible. Although materials like ion interchange membranes and electrode materials are typically the key issues under investigation, we have tried a different strategy based on exploring the physiology electroactive microorganism using *Geobacter sulfurreducens* as a model. We have shown that microbial electroactivity can be induced in planktonic cells even in absence of any electrode during the growth period. This was possible just by culturing the cells under salt conditions. Proteomics analysis demonstrated the osmoadaptation response of the bacteria by adjusting the transport of inorganic and organic species as well as strong changes this membrane structure due to the presence of a shift EPS matrix, mainly enriched in the C-type cytochrome OmcS. Interestingly, EPS wiring matrix seem to actually participate in plugging planktonic *Geobacter* cells into electrodes suggesting novel applications where a controlled electroactive performance is required. This finding predicts suitable electroactive behaviour of *G. sulfurreducens* in devices like Microbial Desalination Cells (MDC) where anodic chamber operates under salt-conditions.

Finally, our research opens the scenario from biofilm-based bioelectrochemistry to planktonic cultures and suggests the key role of the EPS in DEET, in contrast with previous studies focus just on membrane cytochromes (Strycharz-Glaven et al., 2011) and conductive nanowires (Malvankar and Lovley, 2014).

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SUPPLEMENTARY INFORMATION

Figure S1. Left: growth curves of *Geobacter sulfurreducens* batch cultures in different NaCl conditions (0 $g.l^{-1}$,5 $g.l^{-1}$ and 10 $g.l^{-1}$ of NaCl, FWM-0, FWM-5 and FWM-10 respectively). Right: picture of cultures of *Geobacter sulfurreducens* grown in FWM-0, FWM-5 and FWM-10 (from left to right).

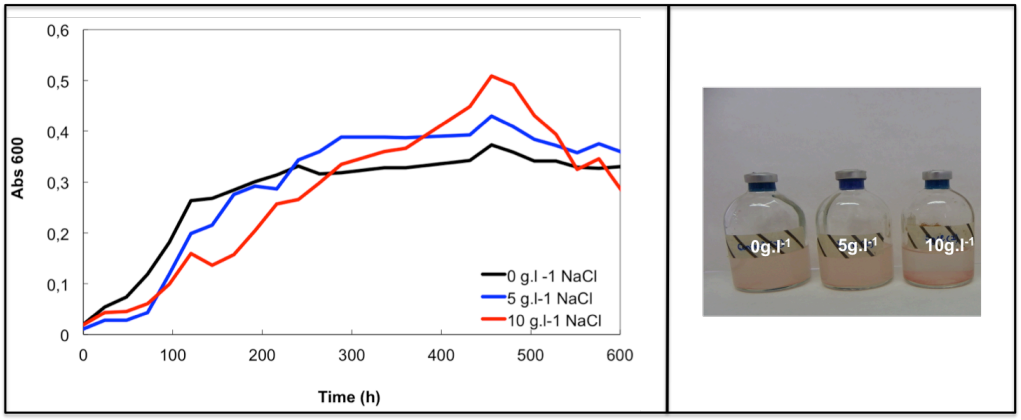

Table S1. Values in mV of Zeta potential of cells cultivated in FWM-0, FWM-5 and FWM-10. Measurement of cells were taken in the working pH7.

Sample/NaCl Condition	Zeta Potential (mV)
FWM-0	-14.6
FWM-5	-20.5
FWM-10	-22.1

Figure S2. The influence of the level of EPS produced by *Geobacter sulfurreducens* on zeta potential values for 0 $g.l^{-1}$, 5 $g.l^{-1}$ an 10 $g.l^{-1}$ NaCl supplemented bacterial growth media.

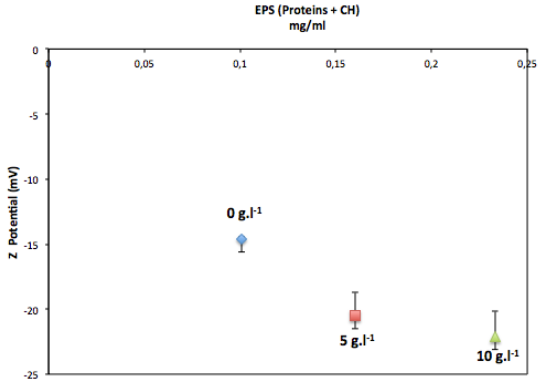

Figure S3. Representation of the changes in the protein profile of a EPS extract from a culture of *G.sulfurreducens* grown with NaCl. Blue bars are the number of proteins with a lower abundance. Red bars, proteins with an increased relative abundance. Categories were assigned based on the function annotated in *Geobacter sulfurreducens* genome.

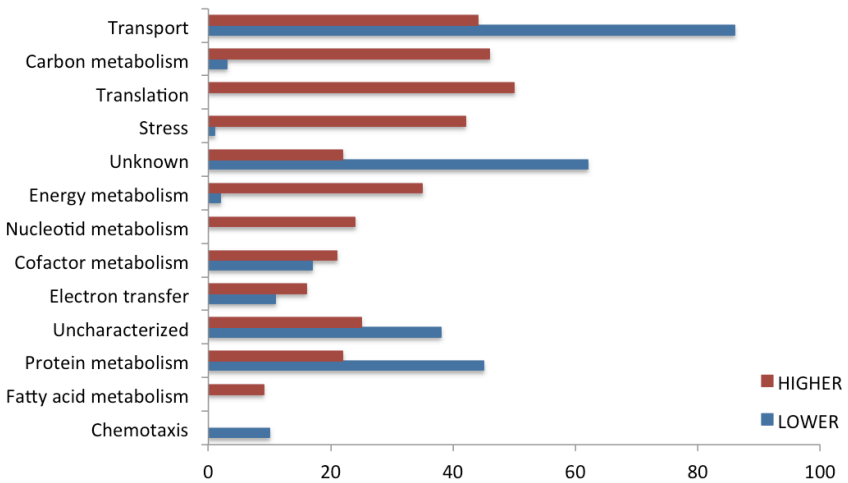

Table S2. Assignment and frequencies (cm^{-1}) of some of the heme-related bands of *G. sulfurreducens* in the NER spectrum.

Symmetry	Frequency (cm^{-1})	Vibrational mode
ν_{10} (B_{1g})	1636	$\nu(C_\alpha C_m)_{asym}$
ν_2 (A_{1g})	1582	$\nu(C_\alpha C_m)_{asym}$
ν_{11} (B_{1g})	1561	$\nu(C_\beta C_\beta)$
ν_{29} (B_{2g})	1402	ν(pyr 1/4ring)
ν_4 (A_{1g})	1371	$\nu(\text{pyr } 1/2\text{ring})_{sym}$

Table S3 and S4. List of groups of proteins affected by the presence of salt in the medium (S3) and groups of proteins from EPS extracts affected by the presence of salt. (S4). The relative abundance fold was obtained by the difference of the unique peptides identified in each group of proteins of both samples. It was considered a difference of 2-fold at least. ID locus GSU and RW are two different entries in UniProt database that both correspond to DL-1 strain *Geobacter sulfurreducens*. KN400 is a strain of *Geobacter sulfurreducens* described to be superior to DL-1 strain in producing electrical current. It has been reported to persist at low frequency in DL-1 cultures even after intensive restreaking of isolated colonies from the DL-1 (Shrestha et al., 2013).

Table S3. Whole-cell proteome

Category	Locus	Symbol	Description	Fold
Energy Metabolism				
	GSU1861	vorA	2-oxoacid:ferredoxin oxidoreductase	2
	GSU1526		Adenine phosphoribosyltransferase	2
	RW64_01370		Alpha/beta hydrolase	4
	GM18_2663		Fumarate lyase	2
	GSU2308	mleA	Malate oxidoreductase, NAD-dependent	5
	GSU0351	nuoN	NADH-quinone oxidoreductase subunit N	2
	GSU1915	dxr	1-deoxy-D-xylulose 5-phosphate reductoisomerase	-2
	GSU2449	sucA	2-oxoglutarate dehydrogenase	-8
	GSU2718	hoxL	Bidirectional NAD-reducing hydrogenase, large subunit O	-3
	KN400_1469		Carbonic anhydrase	.2

	GSU1641	cydB	Cytochrome bd menaquinol oxidase, subunit II	.2
	GSU0346	nuoI1	NADH-quinone oxidoreductase subunit I	-2
	KN400_2470		NADPH oxidoreductase	-2
	KN400_1753		Radical SAM domain iron-sulfur cluster-binding oxidoreductase	-4
	GSU1349	cysP	Sulfate ABC transporter, periplasmic sulfate-binding protein	-3
Translation	GU3612	rpsL	30S ribosomal protein S12	2
	GSU0922		ABC transporter, ATP-binding protein	3
	GSU0177		Acetyltransferase	2
	GSU2870	rpmG	50S ribosomal protein L33	2
	GSU3373	rsmB	Ribosomal RNA small subunit methyltransferase B	3
	GSU0666	rpsR	30S ribosomal protein S18	-2
	GSU1156	asnS	Asparagine--tRNA ligase	-2
	KN400_0969		NHL repeat domain protein	-4
	GSU1516	infC	Translation initiation factor IF-3	-2
Environmental Stress	GSU0538	hspA	ATP-independent chaperone, alpha-crystallin/Hsp20 family	3
	GSU3213	obg	ATP-independent chaperone, alpha-crystallin/Hsp20 family	4

	GSU3161		Methionine sulfoxide reductase A	3
	GSU0856	htpX	Protease HtpX homolog	1
	KN400_0340		Sigma-54-dependent transcriptional response regulator	-2
	KN400_2094		[acyl-]glycerolphosphate acyltransferase	2
Lipid&Phospholipid Metabolism	KN400_2362		SGNH-hydrolase lipoprotein	2
	GSU2762	glpK	Glycerol kinase	-3
	KN400_0433		3-oxoacyl-(Acyl carrier protein) synthase-related protein	-2
Signal Transduction				
	GSU2511		Diguanylate cyclase	2
	GSU1004	gnfL	Histidine kinase	2
	KN400_0792		Histidine kinase	4
	KN400_2512		Histidine kinase	4
	KN400_2859		Histidine kinase	2
	KN400_0858		Response regulator, PilZ domain-containing	2
	KN400_0861		Histidine kinase O	-2
	KN400_3090		PAS domain protein	-2
	KN400_2580		PATAN domain GTPase-activating protein, putative	-2
	GSU1655		Response receiver sensor histidine kinase response	-2

			regulator	
	KN400_3320		Response receiver-modulated diguanylate cyclase	-2
	KN400_1289		Response regulator sensor, GAF domain-containing	-2
	KN400_0989		Sensor cyclic diguanylate phosphodiesterase	-4
	KN400_1965		Sensor histidine kinase cyclic nucleotide phosphodiesterase	-3
	KN400_0969		NHL repeat domain protein	-4
Aminoacid Metabolism				
	GSU2206	rpsT	30S ribosomal protein S20	2
	GSU3093	rpsU2	30S ribosomal protein S2	5
	GSU3006	cbiA	Cobyrinic acid a,c-diamide synthase	2
	GSU3095	hisF	Imidazole glycerol phosphate synthase subunit HisF	3
	GSU3097	hisH	Imidazole glycerol phosphate synthase subunit HisH	2
	GSU3162	ltaA	L-allo-threonine aldolase, stereospecific	2
	GSU3057	nfnA	NADH-dependent ferredoxin:NADP+ oxidoreductase	5
	GSU1028	aguB	N-carbamylputrescine amidohydrolase	4
	GSU3374	rpe	Ribulose-phosphate 3-epimerase	2
	GSU2292	ald	Alanine dehydrogenase	-4
	GSU0375	gcvT	Aminomethyltransferase	-4
	KN400_0199		Cupin superfamily barrel domain protein	-2
	GSU2462	metX	Homoserine acetyltransferase	-2
	KN400_1136		Intracellular protease, PfpI family, putative	-2

	GSU2431	nfeD	Membrane-bound serine protease NfeD, long form	-3
	GSU3303	mceE	Methylmalonyl-CoA epimerase	-3
Cofactor Biosynthesis				
	GSU0454	mqnD	1,4-dihydroxy-6-naphthoate synthase	2
	GSU2683	panE	2-dehydropantoate 2-reductase	2
	GSU0106		Chromosome partitioning protein ParA	2
	GSU2999	cbiC	Cobalt-precorrin-8X methylmutase	3
	GSU2995	cbiL	Cobalt-sirohydrochlorin C20-methyltransferase	2
	GSU0442	mqnC-1	Dehypoxanthinylfutalosine cyclase, putative	3
	GSU0484	folE	GTP cyclohydrolase FolE2	3
	GSU3147	mobB	Molybdopterin-guanine dinucleotide biosynthesis protein	2
	GSU2011	nifS	Nitrogen fixation iron-sulfur cluster assembly cysteine desulfurase	3
	GSU1934	coaX	Type III pantothenate kinase	3
	GSU0867	ubiE	Ubiquinone/menaquinone biosynthesis C-methyltransferase	5
	GSU2017	mqnA	Futalosine synthase, putative	2
	GSU1935	birA	Biotin operon repressor, biotin--acetyl-CoA carboxylase ligase	-2
	GSU0143	cinA	CinA-like protein	-3
	GSU3312	hemH	Ferrochelatase	-3

	KN400_2237		Nicotinate phosphoribosyltransferase	-2
	KN400_0512		Pyridoxal-5'-phosphate-dependent chorismate-binding enzyme	-3
	GSU2521	yedF	Selenium metabolism protein YedF	-3
	GSU0654	thiF-1	Thiamin biosynthesis thiocarboxylate synthase	-3
	GSU3194	thiL	Thiamine-monophosphate kinase	-3
Nucleotide Metabolism				
	GSU1759	purN	Phosphoribosylglycinamide formyltransferase, folate-dependent	2
	GSU2194	guaA	GMP synthase [glutamine-hydrolyzing]	-2
	GSU1403	rluB	Pseudouridine synthase	-4
	GSU3106	thyX	Thymidylate synthase ThyX	-2
	KN400_2190		UDP-2-acetamido-2,6-dideoxy-beta-L-mannose 2-epimerase	-2
Cell envelope				
	KN400_2830		D-alanine--D-alanine ligase	2
	KN400_0113		PilZ domain protein	2
	GSU2367	lptD	LPS-assembly protein LptD	-3
	KN400_2548		LysM domain protein	-3
	GSU3066	ddl	D-alanine--D-alanine ligase	-2
Chemostaxis				
	GSU2220	cheW40H-	Scaffold protein CheW associated with MCPs of class	2

		1	40H	
	GSU2038	pilY1-2	Type IV pilus assembly protein PilY1	-4
	GSU2037	fimU	Type IV pilus minor pilin FimU	-2
	GSU2035	pilW-2	Type IV pilus minor pilin PilW	-2
Electron transfer				
	GSU2927		Cytochrome C	2
	RW64_11590		Cytochrome peroxidase	3
	GSU2797	etfB	Electron transfer flavoprotein subunit beta	2
	GSU0772		Flavodoxin	2
	RW64_18705		Riboflavin synthase subunit alpha	2
	RW64_08785		NADH dehydrogenase	2
	GSU0357		Cytochrome C	-3
	GSU2811		Cytochrome c	-5
	GSU2927	dhc2	Cytochrome c, 2 heme-binding sites	-2
	GSU2503	omcT	Cytochrome c, 6 heme-binding sites	-5
	KN400_0325		Cytochrome c-552	-9
	KN400_0153		Flavodoxin, putative	-3
Carbohydrates				
	GSU2264	lpxA	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase	2
	GSU2365	rmlD	dTDP-4-dehydrorhamnose reductase	3
	RW64_15925		Fructose-bisphosphate aldolase	2

	GSU2358	treZ	Malto-oligosyltrehalose trehalohydrolase	4
	GSU3254		Mannose-1-phosphate guanylyltransferase	2
	GSU2087	gmhA	Phosphoheptose isomerase	3
	KN400_0279		Phospholipase, patatin family, putative	4
	GSU3256	galT	Galactose-1-phosphate uridylyltransferase	2
	GSU1023	glgA-1	Glycogen synthase	-5
	GSU1805	glmM	Phosphoglucosamine mutase	-3
	GSU1524	pcm	Protein-L-isoaspartate O-methyltransferase	-2
	GSU2266	lpxD	UDP-3-O-acylglucosamine N-acyltransferase	-4
	GSU2241	uge	UDP-galacturonate 4-epimerase	-3
Protein metabolism				
	GSU1790	lon-2	Lon protease	3
	GSU0129	def-1	Peptide deformylase	4
	GSU1873	pepF	Oligoendopeptidase F	-7
	KN400_0793		Organic solvent tolerance ABC transporter, periplasmic substrate-binding protein	-3
	KN400_3292		Peptidase, U32 family	-3
	GSU0969	ctpA-1	Periplasmic carboxy-terminal processing protease	-3
	GSU0820	sppA	Signal peptide peptidase SppA	-4
	KN400_3304		Zinc-dependent peptidase, M16 family	-2
Transport&Binding Proteins				
	GSU1165	ptsP	GAF domain phosphoenolpyruvate--protein	3

		phosphotransferase	
KN400_1581		Mechanosensitive ion channel family protein	2
GSU2269	lolE	Lipoprotein release ABC transporter, membrane protein	2
GSU2481	kdpB	Potassium-transporting ATPase B chain	2
KN400_0779		ABC transporter, membrane protein	-3
KN400_1425		ABC transporter, periplasmic substrate-binding protein, 1 heme-binding site	-7
GSU3405		Aminoacid ABC transporter	-2
GSU1156	asnS	Asparagine--tRNA ligase	-2
GSU1731	livG	Branched-chain amino acid ABC transporter	-3
GSU1735	livK-2	Branched-chain amino acid ABC transporter, periplasmic aminoacid-binding protein	-7
GSU0739	ehrA-1	Ech-hydrogenase-related complex, NuoL-like integral membrane subunit	-3
KN400_2604		Efflux pump, RND family, inner and outer membrane proteins	-5
KN400_0362		Efflux pump, RND family, inner membrane protein	-2
KN400_0360		Efflux pump, RND family, membrane fusion lipoprotein	-4
GSU2781		Efflux pump, RND family, membrane fusion protein	-6
GSU2823	ybhG	Efflux pump, RND family, membrane fusion protein	-2
KN400_2641		Efflux pump, RND family, outer membrane lipoprotein	-7
KN400_0359		Efflux pump, RND family, outer membrane protein	-4

	GSU0689	hpnN	Efflux pump, RND superfamily	-5
	KN400_2924		Ligand-gated TonB-dependent outer membrane channel	-12
	GSU1889	lptA	Lipopolysaccharide ABC transporter, periplasmic protein LptA	-3
	RW64_12140		Macro domain-containing protein	-2
	KN400_1305		Metal ion efflux pump, RND family, inner membrane protein	-2
	KN400_1304		Metal ion efflux pump, RND family, membrane fusion protein	-6
	KN400_2076		TRAP proton/solute symporter, periplasmic substrate-binding protein	-2
	GSU1779	pulP	Type II secretion system protein PulP	-2
Unknown function				
	GSU1859	vorC	2-oxoacid:ferredoxin oxidoreductase subunit gamma	2
	GSU1517	rpmI	50S ribosomal protein L35	2
	RW64_15340	ftsY	Cell division protein FtsY	2
	GSU2256		Heptosyltransferase	2
	RW64_16335		Homocysteine methyltransferase	3
	RW64_10765		Hydrogenase assembly protein HupF	2
	GSU0971		Hydrolase	4
	KN400_1963		Nucleotide cyclase, HAMP and GGDEF-related domain-containing	2

KN400_0203		Peptidyl-prolyl cis-trans isomerase	2
KN400_1861		Phosphatase/phosphohexomutase-related hydrolase	3
KN400_0806		Pirin family protein	3
KN400_2177		PSP1 superfamily protein	3
KN400_2177		PSP1 superfamily protein	4
RW64_16070		Radical SAM protein	2
GSU1999		RNA-binding protein	2
KN400_3264		SAM-dependent methyltransferase	2
KN400_2123		Sensor transcriptional regulator, PAS and LytTR domain-containing	2
KN400_2606		Transcriptional regulator, TetR family	2
KN400_1796		Zinc metalloendopeptidase M23 domain protein	2
KN400_2081		Zinc protease PmbA, putative	3
RW64_19145		Acriflavine resistance protein B	-3
GSU3020		Acyltransferase, left-handed parallel beta-helix (Hexapeptide repeat) family, lipoyl attachment domain-containing	-2
KN400_2137		Aldolase domain protein	-3
KN400_1997		Aminotransferase, AHBA_syn family	-5
RW64_18775		AMP-binding protein	-2
GSU1273	carA	Carbamoyl phosphate synthase small subunit	-3
KN400_2272		Cation-translocating P-type ATPase	-3

GSU1130	smc	Chromosome partition protein Smc	-2
GSU1387	cse4	CRISPR processing complex protein CasC	-6
RW64_01360		Cupin	-2
KN400_2279		Cytidylate kinase-like domain protein	-2
GSU3424		Dihydrolipoamide dehydrogenase	-2
RW64_17610		Dihydroorotate dehydrogenase	-2
RW64_04140		Exopolyphosphatase	-2
GSU0585	ycgM	Fumarylacetoacetate hydrolase family protein	-2
KN400_2159		GAF domain protein	-2
GSU3023		Glycosyltransferase and TPR domain protein	-2
KN400_0256		HEAT-like repeat-containing protein	-3
KN400_0334		Helix-turn-helix XRE domain protein	-2
RW64_13320		Hydrogenase	-3
GSU1337		Lipoprotein	-3
KN400_0039		Lipoprotein	-2
KN400_1580		Lipoprotein	-2
KN400_3067		Lipoprotein	-2
RW64_00455		Magnesium transporter	-2
KN400_1184		Metal-dependent hydrolase, beta-lactamase superfamily	-2
KN400_1475		Metal-dependent phosphoesterase, PHP family	-2
GSU2493		NHL repeat domain protein	-7

	KN400_1991	Nucleotidyltransferase, CBS domain pair and CBS domain pair-containing	-7
	KN400_1130	Outer membrane protein	-4
	RW64_14305	Peptidase M16	-2
	KN400_0351	Peptidyl-prolyl cis-trans isomerase	-2
	KN400_0329	Peptidylprolyl isomerase	-2
	GSU2106	PglZ domain protein	-2
	KN400_0962	Phage protein D	-5
	KN400_1971	Repeat-containing protein	-6
	RW64_18100	RND transporter	-2
	GSU2626	SAM-dependent methyltransferase	-2
	GSU2792	SAM-dependent methyltransferase	-2
	SE37_08735	Signal peptidase I	-4
	KN400_3255	Thiolase	-3
	KN400_2010	TPR domain lipoprotein	-5
	GSU1190	tRNA 2-selenouridine synthase	-2
	GSU1900	Uncharacterized protein	-3
	GSU0924	Uncharacterized protein	-2
	KN400_2455	Undecaprenyl-phosphate glycosyltransferase	-3
	GSU3586	YVTN family beta-propeller domain protein	-11
Hypothetical Proteins			
	GSU0562	GSU0562 Uncharacterized protein	2

GSU0564		Uncharacterized protein	2
GSU2105		Uncharacterized protein	2
GSU2109		Uncharacterized protein	2
KN400_0727		Uncharacterized protein	2
KN400_1058		Uncharacterized protein	2
KN400_1572		Uncharacterized protein	2
KN400_1576		Uncharacterized protein	2
KN400_1772		Uncharacterized protein	3
KN400_2036		Uncharacterized protein	4
KN400_2271		Uncharacterized protein	2
KN400_2319		Uncharacterized protein	3
KN400_2508		Uncharacterized protein	2
KN400_2946		Uncharacterized protein	2
GSU0020	yrdA	Uncharacterized protein	3
KN400_1162		Adenosine kinase	-3
GSU0280		Fis family transcriptional regulator	-7
KN400_1162		Fis family transcriptional regulator	-4
RW64_14845		Fis family transcriptional regulator	-2
GSU1011		TPR domain protein	-2
GSU2476		TPR domain protein	-3
GSU2508		TPR domain protein	-9

KN400_1143	Uncharacterized protein	-2
KN400_1485	Uncharacterized protein	-4
GSU0992	Uncharacterized protein	-17
GSU2199	Uncharacterized protein	-7
GSU3503	Uncharacterized protein	-2
KN400_0770	Uncharacterized protein	-5
KN400_0868	Uncharacterized protein	-3
KN400_0770	Uncharacterized protein	-2
KN400_0954	Uncharacterized protein	-3
KN400_0955	Uncharacterized protein	-4
KN400_0960	Uncharacterized protein	-2
KN400_0967	Uncharacterized protein	-9
KN400_0968	Uncharacterized protein	-5
KN400_1186	Uncharacterized protein	-8
KN400_1187	Uncharacterized protein	-2
KN400_1992	Uncharacterized protein	-6
KN400_1187	Uncharacterized protein	-6
KN400_2583	Uncharacterized protein	-3
KN400_2690	Uncharacterized protein	-2
KN400_3071	Uncharacterized protein	-3
KN400_3077	Uncharacterized protein	-2

Table S4. EPS proteome

Category	Locus	Symbol	Description	Fold
Energy Metabolism				
	GSU2707	ackA	Acetate kinase	8
	SE37_09440		AMP-binding protein	2
	GSU0994	fumB	Fumarate hydratase, class I	1
	GSU0674	hcp	Hydroxylamine reductase	3
	RW64_10940		NADH dehydrogenase	2
	RW64_10950		NADH dehydrogenase	8
	GSU1818	apgM	Probable 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	2
	GSU2428	pyc	Pyruvate carboxylase	1
	GSU0113	atpD	ATP synthase subunit beta	7
	GSU0340	nuoC	NADH-quinone oxidoreductase subunit C	2
	RW64_15545		Diguanylate phosphodiesterase	-2

Translation				
	GSU1463	aspS	Aspartate--tRNA(Asp/Asn) ligase	8
	GSU2860	fusA	Elongation factor G (Fragment)	2
	GSU1752	epf	Elongation factor P	2
	GSU1920	tsf	Elongation factor Ts	3
	GSU0578	glyQ	Glycine--tRNA ligase alpha subunit	2
	GSU2209	leuS	Leucine--tRNA ligase	2
	GSU2271	lysS	Lysine--tRNA ligase	3
	GSU1520	pheT	Phenylalanine--tRNA ligase beta subunit	4
	GSU0037	serS	Serine--tRNA ligase	2
	GSU3136	ileS	Isoleucine--tRNA ligase	7
	GSU1460	proS	Proline--tRNA ligase	2
	GSU1515	thrS	Threonyl-tRNA synthetase	8
Environmental Stress				
	GSU3340	groL	60 kDa chaperonin	13
	GSU0033	dnaK	Chaperone protein	6
	RW64_02245		Erythromycin biosynthesis sensory transduction	5

			protein	
	RW64_02870	htpG	Heat shock protein 90	5
	RW64_06995		Penicillin-binding protein	6
	RW64_10990	tpx	Thioredoxin peroxidase	2
	RW64_02590		Universal stress protein	-4
Lipid&Phospholipid Metabolism				
	GSU0290	fabH-1	3-oxoacyl-[acyl-carrier-protein] synthase 3	2
	RW64_10685	fabH	3-oxoacyl-ACP synthase	3
	RW64_00860		Acetyl-CoA carboxylase	3
	GSU1402	accA	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	3
AA biosynthesis				

RW64_00340	leuA	2-isopropylmalate synthase	3
RW64_20020		3-hydroxyisobutyrate dehydrogenase	4
RW64_00325	leuC	3-isopropylmalate dehydratase	2
GSU1911	ilvB	Acetolactate synthase catalytic subunit	2
GSU0151	argD	Acetylornithine aminotransferase	4
GSU0153	argG	Argininosuccinate synthase	5
GSU1242		Aspartate aminotransferase	6
GSU0158		Diaminopimelate decarboxylase	4
GSU3211	proA	Gamma-glutamyl phosphate reductase	2
GSU1693	hom	Homoserine dehydrogenase	2
GSU3098	hisB	Imidazoleglycerol-phosphate dehydratase	2
GSU1909	ilvC	Ketol-acid reductoisomerase	7
GSU2874	argC	N-acetyl-gamma-glutamyl-phosphate reductase	2
GSU2541		Pyrroline-5-carboxylate reductase	2
GSU2379	trpB	Tryptophan synthase beta chain	3
GSU2291	aroF	AroF protein	-3

	GSU2572	cysE-1	Hexapeptide transferase	-2
Cofactor Biosynthesis				
	GSU3299		Biotin-dependent acyl-CoA carboxylase, carboxyltransferase subunit	4
	RW64_06940		Hydroxyacid dehydrogenase	2
	GSU1827	nadB	L-aspartate oxidase	2
	GSU0130	fmt	Methionyl-tRNA formyltransferase	2
	GSU1124	coaBC	Phosphopantothenoylcysteine decarboxylase	9
	GSU3285	hemC	Porphobilinogen deaminase	2
	GSU3453	hemE	Uroporphyrinogen decarboxylase	3
	GSU0135	hemB	Delta-aminolevulinic acid dehydratase	-2
	GSU3378	glnE	Glutamate-ammonia-ligase adenylyltransferase	-2
	GSU3284	hemA	Glutamyl-tRNA reductase	-2
	RW64_07855		Methylmalonyl-CoA mutase	-2
Chemotaxis				
	RW64_09050		Flagellar motor protein MotA	-3
	RW64_13020		Flagellar motor protein MotB	-17
	RW64_00905		Pilus assembly protein PilQ	-3
	RW64_00955		Pilus assembly protein PilY	-3

Nucleotide Metabolism			
	GSU3308	purA	Adenylosuccinate synthetase	8
	GSU1271	pyrB	Aspartate carbamoyltransferase	4
	GSU0609	purH	Bifunctional purine biosynthesis protein	3
	GSU1272	pyrC	Dihydroorotase	2
	GSU1755	pyrD	Dihydroorotate dehydrogenase	2
	GSU1110	ndk	Nucleoside diphosphate kinase	2
	GSU2091	purC	Phosphoribosylaminoimidazole-succinocarboxamide synthase	2
	GSU1758	purM	Phosphoribosylformylglycinamidine cyclo-ligase	3
	GSU1634		Phosphoribosylformylglycinamidine synthase	2
	GSU1717		Sulfate adenylyltransferase	2
Electron Transfer			
	GSU2932		Cytochrome B6	6
	RW64_05810		Cytochrome C	8
	RW64_05315		Electron transfer flavoprotein	4

	GSU2504	OmcS	Cytochrome C	4
	RW64_07815		Lactamase	3
	RW64_03535		Cytochrome C	-2
	RW64_12535		Cytochrome C	-2
	RW64_12545		Cytochrome C	-4
	GSU2813	ccpA	Cytochrome C peroxidase	-1
	GSU0720		Desulfoferrodoxin	-4
Carbon Metabolism				
	GSU2286	eno	Enolase	9
	GSU1651	fbp	Fructose-1,6-bisphosphatase class 1	4
	GSU2706	pta	Phosphate acetyltransferase	3
	RW64_00830		Phosphoglucomutase	7
	GSU0580		Pyruvate phosphate dikinase	7
	GSU1607	glyA	Serine hydroxymethyltransferase	9

Transport&Binding Proteins	GSU3627	tpiA	Triosephosphate isomerase	3
	GSU0371		Alpha-glucan phosphorylase	-4
	RW64_17715		ABC transporter substrate-binding protein	2
	RW64_08590		Amino acid ABC transporter substrate-binding protein	5
	A0A0C1TS96		Amino acid-binding protein	2
	RW64_01035		C4-dicarboxylate ABC transporter substrate-binding protein	2
	GSU3291	hppA	K(+)-insensitive pyrophosphate-energized proton pump	2
	GSU0501	bamA	Outer membrane protein assembly factor	2
	GSU3406		Polar amino acid/opine ABC transporter, periplasmic amino acid-binding protein	7
	GSU2617	secD	Preprotein translocase subunit	4
	RW64_16355		RND transporter	3

RW64_13065		Transporter	2
GSU1793	tig	Trigger factor	6
RW64_04925		Tungsten ABC transporter substrate-binding protein	6
RW64_00305		ABC transporter substrate-binding protein	-8
GSU2695		Efflux pump, RND family, outer membrane protein	-9
RW64_06250		Ligand-gated channel	-8
GSU1445		Ligand-gated TonB-dependent outer membrane channel	-2
RW64_19410		Mechanosensitive ion channel protein MscS	-2
RW64_17800		Mechanosensitive ion channel protein	-2
GSU1229		Outer membrane channel lipoprotein, putative	-6
RW64_13460		Outer membrane channel protein	-2
GSU1482		Protein CyaE	-9
RW64_18100		RND transporter	-6
RW64_19860		RND transporter	-13
GSU1486	tatC	Sec-independent protein translocase protein TatC	-2
RW64_04905		Multidrug transporter	-7
RW64_00255		Sugar ABC transporter substrate-binding protein	-3

Protein Metabolism				
	GSU1875	ahcY	Adenosylhomocysteinase	3
	RW64_19385		Amino acid dehydrogenase	6
	GSU0270	glmS	Glutamine--fructose-6-phosphate aminotransferase	2
	RW64_11130		Glycine dehydrogenase	5
	GSU0305	hypB	Hydrogenase nickel incorporation protein	2
	GSU0716		Peptidase C14	5
	RW64_10885		Peptidase	8
	RW64_17525		Peptidase S41	5
	GSU2537	speA	Biosynthetic arginine decarboxylase	-5
	GSU0923	lon-1	Lon protease	-5
	RW64_04815		Peptidase C1	-13
	GSU0015		Peptidylprolyl isomerase	-3
Unknown function				
	A0A0C1U5A7		Acyltransferase	2
	RW64_17835		Adenylylsulfate reductase	2
	GSU1799		Aspartokinase	2
	GSU0215	folD-1	Bifunctional protein	2
	GSU3300		Biotin-dependent acyl-CoA carboxylase, biotin	2

		carboxylase subunit	
GSU2605	cmk	Cytidylate kinase	3
RW64_14220		FeS-binding protein	2
GSU1738	iorB-1	Indolepyruvate oxidoreductase subunit beta	2
GSU1602	fabD-2	Malonyl CoA-acyl carrier protein transacylase	2
GSU0029		Nitrilase/amidohydrolase superfamily protein, class 5	4
RW64_03135		Peptidylprolyl isomerase	3
RW64_14115		Peroxiredoxin	4
RW64_01785		Protein-disulfide isomerase	3
GSU3085	yqfO	Putative GTP cyclohydrolase 1 type 2	3
GSU2516		Rhodanese homology domain pair protein	2
GSU2612		Rubrerythrin	3
GSU1833	trpS	Tryptophan--tRNA ligase	3

	RW64_02250		UDP-N-acetyl-D-glucosamine dehydrogenase	2
	RW64_14015		AsmA family protein	-3
	RW64_04870		Glycoside hydrolase family 43	-4
	RW64_13565		Hydrogenase	-8
	RW64_16110		Membrane protein	-3
	GSU2431	nfeD	Membrane-bound serine protease NfeD, long form	-3
	RW64_03660		Nitrite reductase	-3
	RW64_03120		Oxidoreductase	-2
	RW64_08720		Oxidoreductase	-2
	GSU3586		YVTN family beta-propeller domain protein	-12
Hypothetical Proteins				
	GSU1089		Iron-sulfur cluster-binding oxidoreductase	3
	A0A0C1R0X8		Uncharacterized protein	2
	GSU3305		Uncharacterized protein	4
	GSU3306		Uncharacterized protein	2
	RW64_06335		Uncharacterized protein	2
	GSU1497	pilA-C	Geopilin domain 2 protein	-3
	GSU2499		Uncharacterized protein	-12

GSU2726	Uncharacterized protein	-6
GSU340	Uncharacterized protein	-3
RW64_00700	Uncharacterized protein	-3
RW64_01940	Uncharacterized protein	-8
RW64_03495	Uncharacterized protein	-4
RW64_04660	Uncharacterized protein	-5
RW64_07270	Uncharacterized protein	-3
RW64_07730	Uncharacterized protein	-3
RW64_13595	Uncharacterized protein	-4
RW64_19985	Uncharacterized protein	-4

Saint-Gobain

Part II. Microbial Electrochemical Applications

Chapter 3. Introduction to Microbial Electrochemical Applications

Chapter 4. Test of a novel MET prototype: set up of the optimal operation conditions

Chapter 5. Desalination by MDC system with pure culture of *Geobacter sulfurreducens*

Chapter 3

Introduction to Microbial Electrochemical Applications

MICROBIAL ELECTROCHEMICAL TECHNOLOGIES: origin and main members of the platform

Microbial electrochemistry is the discipline focus on the interactions between living microbial cells and conductive materials (electrodes). Although the bacterial capacity to link the oxidation of organic compound to the reduction of electrodes was known since the origins of the XX century (Potter, M.C., 1910a, 1910b), it took 50 years to develop the first Microbial Fuel Cell device (MFC)(Berk, R.S. and Canfield, J.H., 1965; Van Hees, W.A., 1965). However, it was not considered a real technological alternative until the beginning of XX century. Indeed, it was in 2001 when the MFCs field went through a revolution whose origin was the key research from Reimers et al. (2001). They demonstrated that electric energy can be harvested from the natural voltage gradient generated between the anoxic zone and the overlaying oxygenic seawater in marine sediments. In addition, it was shown that this gradient was due to the presence of electroactive microorganisms (see Chapter 1). Reimers et al. work triggered the beginning of a new MFC technology era.

A MFC device is basically a system in which microorganisms catalyse the conversion of chemical energy into electrical energy. The microorganisms oxidize the organic matter (electron donor) while an insoluble electron acceptor (electrode) is subsequently reduced. The finding of microorganisms capable of reducing the electrode without a soluble electron transfer (See chapter 1), freed MFC technology from the need of an expensive and environmentally dangerous, exogenous soluble electron shuttle (Debabov, 2008; Logan and Rabaey, 2012; Lovley, 2006a; Rabaey and Verstraete, 2005). Furthermore, the ubiquity of electroactive bacteria in a vast diversity

of habitats evidences the versatility of MFC technology.

Since 2001, and in addition to MFCs, a large variety of innovated systems related to MFCs have been developed, such as microbial electrolysis cell (MECs) for electrochemical production of H_2 (Liu et al., 2005), microbial desalination cells (MDCs) for water desalination (Cao et al., 2009; Jacobson et al., 2011), microbial electroremediating cells (MERCs) for restoring polluted environments (Rodrigo et al., 2014) or even microbial electrosynthesis cells (MES) (Rabaey and Rozendal, 2010; Rabaey et al., 2011). All these technologies integrate the platform of **Microbial Electrochemical Technologies (METs)** (Schröder et al., 2015).

MICROBIAL FUEL CELL (MFC) AND MICROBIAL ELECTROLYSIS CELL (MEC)

Concept and Design

The MFC is the main device that uses electroactive microorganisms to produce electric energy from the oxidation of organic matter. The basic design of MFC consists in two chambers (anodic and cathodic) separated by an ion exchange membrane. In the anodic chamber, the organic matter (electron donor) is oxidised by bacteria producing CO_2, electrons and protons. These electrons are transferred from the anode to the cathode by an external electric circuit, while protons are transported to the cathodic chamber across the ion exchange membrane by a concentration gradient. In the cathodic chamber, oxygen (the most common electron acceptor) accepts those electrons and, in combination with the protons, it is reduced to water on the cathode surface (Figure 1).

The capacity of electroactive microorganisms for delivering electrons at low potential, provides the potential difference

between anode and cathode in order to impulse the electrons. The power output that can be obtained from the system would depend on the potential difference (V) in combination with electrical current production (I), as follows (Logan, 2008; Rabaey and Verstraete, 2005):

$$P=I.V$$

The main advantage of MFCs is not only the fact of producing electricity but also the availability of a potential difference that can be used to produce work. These electrons can be perfectly used to run electrosynthesis, for instance hydrogen synthesis. When studying the V achieved by bacteria in MFC, it was demonstrated how, by increasing this electrochemical potential with an external additional voltage, it was possible to produce hydrogen at the cathode directly from the oxidized organic matter. More than 90% of the protons and electrons produced by the bacteria from the oxidation of acetate in the anode were recovered as hydrogen gas. Although an external applied voltage is required, it is in an order of magnitude lower than that required for water electrolysis, what demonstrated the potential of this technology. This bio-electrochemically assisted microbial system (Liu et al., 2005) was later renamed as the currently-known Microbial Electrolysis Cell (MEC).

MEC design also consists of two chambers separated by an ion exchange membrane (Figure 1). The additional voltage between anode and cathode is applied in a constant way over time with a power supply or a potentiostat. The latter electronic equipment maintains the potential of the working electrode (anode) constant with respect to the reference electrode by adjusting the current at the auxiliary electrode or counter-electrode (cathode).

Although conventional MFC and MEC design is associated to this two-chambered configuration, it is also possible to operate in a single-chamber mode. In this configuration working electrode and counter-electrode share the same electrolyte, eliminating the need and high cost of an ion exchange membrane. Thus, simplifying the reactor architecture design could lead to reducing capital costs in a considerably way (Call and Logan, 2008; Hu et al., 2008). Furthermore, the removal of the membrane reduces ohmic resistance improving current densities (Call and Logan, 2008).

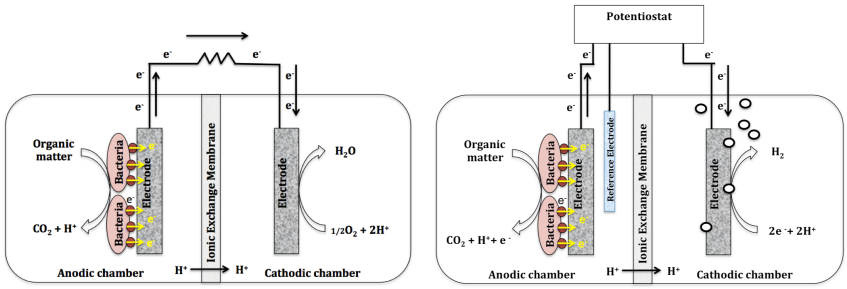

Figure 1. Scheme of two-chambered MFC (left) and MEC (right) both using organic matter as electron donor.

Materials and Construction

The electrodes materials used in METs are conductive solids with a key effect on METs performance. For instance, the electrode material usually determines the power loss of fuel cell in terms of internal resistance (Oh and Logan, 2005). In addition, other parameters such as longevity of electrodes and cost are important to take into account.

Electrodes materials should be not only conductive but also biocompatible (the same for biocathodes). The anode must have high specific area, high porosity and rugosity and must be

chemically stable in the reactor solution (Logan, 2008; Logan et al., 2006). In the case of metal anodes, it is common to use noncorrosive stainless steel mesh avoiding the use of other metals with toxic effects on microorganisms. However, the most extensively used material is carbon. Because, it is comparatively inexpensive and it is available in different several formats as fibrous material (carbon fibre, paper, foam or cloth), as compact graphite plates, rods, granules and also more compact forms of carbon like reticulated vitreous carbon (RVC)(Jourdin et al., 2014; Lepage et al., 2012). In general, for all kind of anode materials, a larger surface area of electrode leads to increased power outputs (Rabaey and Verstraete, 2005). In addition to the electrode material election, a common strategy employed is the chemical functionalization of the anode surface in order to improve bacterial adhesion (i.e. one of the main factors affecting anode performance) and hence, METs performance. (Feng et al., 2010; Kumar et al., 2013; Ramachandran et al., 2015; Saito et al., 2011).

Regarding to cathode material, carbon materials are also the most common ones. Oxygen is the standard electron acceptor in MFC due to its high oxidation potential, vast availability, sustainability and low cost. However, the poor kinetics of its reduction reaction at neutral pH and low temperatures (HaoYu et al., 2007) make the need for a catalyst. Platinum is the best-known oxygen reduction catalyst, however it is not practical for applications due to its high cost and sensitivity to biological and chemical fouling. Therefore, the choice of the cathode material becomes a critical issue that greatly affects METs performance and it depends on the application (Logan et al., 2006).

In MFCs and MECs, when cathodes are made of carbon, the most commonly employed catalysts for an enhanced power output

are the chemicals one. Among them, ferricyanide is the most utilized due to its faster reduction kinetics, its unlimited solubility and because it does not require the use of precious metals on the cathode like platinum (Rismani-Yazdi et al., 2008). Other catalysts that improve cathode reaction are microorganisms (Kim et al., 2015). The concentration of these catalysts as well as the area of the electrode affects the power output. However it is possible to design a MFC that does not require that the cathode to be placed in water. In hydrogen fuel cells, the cathode is bonded directly to the ion exchange membrane so that oxygen in the air can directly react at the electrode(Liu and Logan, 2004). This technique was successfully used in single chamber MFC (Sevda et al., 2013)

APPLICATIONS OF METs

The main characteristic of MFC devices is the production of electricity coupled to wastewater. However, the power output reached so far, make industrial applications still not available. Due to this limitation, the electricity storage in rechargeable devices as capacitors (Liang et al., 2011) has been suggested. On the other hand, promising performances on laboratory scale have presented the MFCs as a suitable option for applications like bioremediation or even biosensing (see below).

With respect to MEC, since its discovery in 2005 (Liu et al., 2005), it has become in a new and promising approach for hydrogen production from organic matter (including wastewater and other renewable resources) (Kapdan and Kargi, 2006; Liu et al., 2005) and therefore, it has been extensively reviewed. The study on MECs includes the analyses of inoculum sources, electrode materials, cell architecture, process performance and energy efficiencies (Liu et al.,

2005, 2010). Compared to classical biological technologies, MECs can overcome thermodynamic limitations and achieve high-yield hydrogen production from wide range of organic matters at relatively mild conditions. This approach greatly reduces the electric energy cost for hydrogen production in contrast to direct water electrolysis (Kadier et al., 2015). Furthermore, it has been also observed that MEC may supposed an alternative application in the field of chemicals synthesis (Cheng et al., 1997; Rozendal et al., 2009; Steinbusch et al., 2010; Zhao et al., 2012a), recalcitrant pollutants removal (Wang et al., 2012; Wen et al., 2013), metal recovery (Heijne et al., 2010; Jiang et al., 2014) and also in biosensors (Zhang and Angelidaki, 2011). MEC achievements as a whole invite for further research in the current challenges and future prospects (Jafary et al., 2015, 2015; Li et al., 2013; Zhang and Angelidaki, 2014).

Energy from Wastewaters

Urban wastewater: in the field of urban wastewater treatment there is an increasing need to minimise the high operational costs, mainly associated with aeration systems and sludge production that account up to the 50 % of the total operational expenses (Wei et al., Aelterman). Taking into account that urban wastewaters contain high amount of organic matter, they become in a potential energy storage that actually represent almost 10-fold the energy invested in treating the wastewaters (Logan et al., 2006). Thus, if METs performances are improved, they appear as a promising technology capable of harvesting the energy contained in urban wastewaters for the production of clean electrical energy. Traditionally, urban wastewaters are processed under anaerobic conditions in order to transform organic matter into acetate and short-chain organic acids.

This acetate is further converted into methane and, although methane can be used as energy source in the wastewater treatment plant, it constitutes a powerful greenhouse-effect gas. One METs ´proposal allows replacing such a methanogen stage by an advanced microbial fuel cell system able to convert acetate into electricity at high rate.

To date, METs technology has been successfully used in lab-scale (Aelterman et al., 2006; Ahn and Logan, 2010; Capodaglio et al., 2013) and pre-pilot scale (Borjas et al., 2015) studies to treat domestic wastewater focussing on organic matter removal and energy production.

Furthermore, METs can accomplish both matter removal and energy production coupled to inorganic nitrogen reduction. It has been demonstrated that Geobacter species located in the cathode, are able to retrieve electrons directly from the poised graphite electrode (biocathode) without hydrogen formation and use these electrons to reduce nitrate to nitrite (Gregory et al., 2004). In this way, it is possible to perform denitrification by microorganisms without any power input but coupled with energy recovery by using a biological anode of a MFC for direct electron delivery (Clauwaert et al., 2007; Cucu et al., 2016).

Based on the same target of a more sustainable technology for wastewater treatment, METs has been incorporated in constructed wetlands (CWs). CWs are proposed as an alternative wastewater treatment in small communities because of their low cost operation and maintenance and low energy requirements. One of the disadvantages of CWs is the surface area per inhabitant being required. However, the coupling of METs and CWs has resulted in a powerful hybrid technology for enhancing the biodegradation rates in

wastewater treatment or for reducing the classical CW dimensions (Wei et al., 2015)

Industrial wastewater: Electrogenic bacteria are able to couple degradation of a diversity of substrates to electricity production (Pant et al., 2010), hence wastewater with a high content of organic matter from a number of industries like food (Durruty et al., 2012; Oh and Logan, 2005; Patil et al., 2009), agricultural (Gálvez et al., 2009), pharmaceutical (Velvizhi and Venkata Mohan, 2011) are good candidates for MET treatment.

Anaerobic characteristic of electrogenic bacteria avoids the energy required for strong aeration in aerobic treatments and, taking into account that MET produces its own amount of energy, cost savings are highly increased (Logan and Rabaey, 2012). However, construction and operation costs of MFC must still be reduced for a real scaling up and industrial application (Franks and Nevin, 2010).

Biosensors: Among the previous mentioned METs applications, there is an alternative research branch that is receiving increasingly more attention: the downscaled METs (Choi, 2015; Jiang et al., 2015; Yoon et al., 2014). Such platform presents advantages not only in relation to fabrication costs (Choi, 2015) but also in terms of use efficiency (higher surface area to volume ratio) (Ren et al., 2014). In comparison to conventional MFCs, miniaturized-METs allow obtaining significantly shorter start-up times and rapid electrical response (Estevez-Canales et al., 2015a; Jiang et al., 2015; Li et al., 2011; Wang et al., 2011). One of the most feasible applications for the downscaled METs are biosensors based on electrochemical detection, as they can perform in situ analysis with high accuracy and stability (Yang et al., 2015). In wastewater technologies, biosensors have been successfully developed for measuring the biological oxygen demand

(BOD) (Peixoto et al., 2011; Zhang and Angelidaki, 2011), acetate (Estevez-Canales et al., 2015b; Li et al., 2011), glucose (Kumlanghan et al., 2007), pH (Uria et al., 2016) as well as toxic compounds (Dávila et al., 2011; Lee et al., 2015a)..

Environmental applications

Harvesting energy from sediments and soil: SMFC:

Benthic or sediment microbial fuel cells (SMFCs) are variants of MFCs that are placed in ecological water bodies. The anode is placed in the sediment and connected through an electrical circuit to a cathode in the overlaying water layer. The energy is obtained by means of several reactions like either the electrochemical oxidation of microbial produced reductans like humic acids, Fe^{2+}, S^{2-} or the microbial oxidation of organics. Most of the SMFCs described so far have been installed in marine sediments, like the described before of Reimers et al. (Reimers et al., 2001). In this environment, the high conductivity of the water reduces the internal cell resistance leading to a higher power production (Logan et al., 2015).

Other variation of SMFC have been located in freshwater natural environments like rice crop soils, where the plant exudates provide a source of organic substrates for the SMFC. In these circumstances, the high electric resistance of the sediment can restrain the transport of ions between anode and cathode and consequently limit SMFC performance. Some authors have overcome this limitation by adding NaCl to the sediment (Hong et al., 2009), changing the soil physical properties by stimulating colloid formation via the addition of silica (agricultural reagent) (Domínguez-Garay et al., 2013) or even locating the anode in a separated water layer (Lee et al., 2015b)

Since SMFC is a system that can continuously generate electricity without significant maintenance, they have being studied for powering of electronic devices (oceanography sensors for temperature, conductivity, oxygen) (Schrader et al., 2016) which are usually located in remote areas or deep waters where replacement or maintenance of power sources is challenging and consumes a large amount of time. Furthermore, electrons coming from SMFC have been used to provide cathodic protection to stainless steel (Orfei et al., 2006).

Bioelectroremediation: MERC:

Bioremediation is a relatively efficient and cost-effective technology for treating polluted soils, yet it finds limitation in bacteria respiration process. Microbial electroremediating cells (MERCs) consist in a variety of bioelectrochemical devices that aim to overcome bacterial electron acceptor limitation and maximize metabolic activity with the purpose of enhancing the biodegradation of a pollutant in the environment (Rodrigo et al., 2014). It has been demonstrated that the presence of the electrode in a polluted sediment increases the bioremediation (Franks and Nevin, 2010; Rooney-Varga et al., 1999). In MERCs anode the degradation of several pollutants, with the consequent production of current, have been studied. Pollutants such as polycyclic aromatic hydrocarbons (Morris and Jin, 2012; Rodrigo et al., 2014; Sherafatmand and Ng, 2015), phenol (Huang et al., 2011a), chlorinated organics (Chun et al., 2013) have been successfully bioremediated. Moreover, herbicides like isoproturon has been completely biodegradated with MERCs devices through new-termed process “bioelectroventing” where, bioremediation under soil-flooded conditions is enhanced by using electrodes as microbial electron sink (Rodrigo Quejigo et al., 2016).

MICROBIAL DESALINATION CELL (MDC)

Introduction

Just 3% out of the total amount of water on the Earth is freshwater while the 97% remaining is seawater and therefore cannot be directly used for drinking. But, due to the increasing industrialization and population growth, the demand for freshwater is further intensified.

In those regions where freshwater remains inaccessible and seawater or brackish water are available, desalination techniques and processes are used. The main desalination technologies employed are distillation, congelation and the membrane technologies (electrodialysis (ED) and reverse osmosis (RO)). Although the last one are less energy-cost, all of them are characterized for being high energy demand (AlMarzooqi et al., 2014; Elimelech and Phillip, 2011; Mathioulakis et al., 2007).

Due to the versatility of the promising MFC technology, its capacity for development is under constant growth. One example is the hybrid concept so-called microbial desalination cell (MDC) that was born with the merge of MFC technology with the ED design (Figure 2) for simultaneous wastewater treatment and bioelectricity production (Cao et al., 2009). MDC become of great interest because it may address wastewater treatment, bieoenergy production and water desalination in a single device, in one step and using the chemical energy storage in wastewaters (ElMekawy et al., 2014; Kim and Logan, 2013; Saeed et al., 2015).

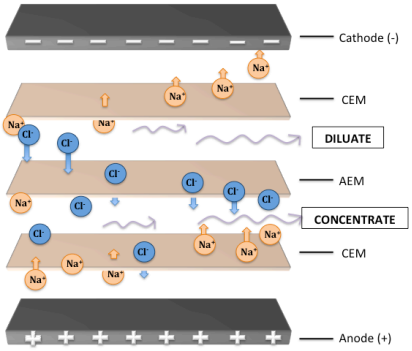

Figure 2. Electrodialysis cell consists on a feed (dilute) compartment and a concentrate (brine) compartment formed by an anion exchange membrane and a cation exchange membrane placed between two electrodes. The membrane process of ED consists on the transport of the salt ions from one solution through ion-exchange membranes to another solution under the influence of an applied electric potential difference.

In order to convert a MFC into a MDC, a third chamber need to be inserted between the anodic and cathodic chambers and separated by an anion exchange membrane (AEM) and a cation exchange membrane (CEM). This third chamber is the desalination chamber of the MDC (Figure 3).

Desalinization process in a MDC is somewhat similar to electrodialysis since is based on transfer of ionic species out of water in proportion to a certain current. However, in a MDC and in contrast to ED, no external electrical energy source is required for separating the ionic species because the current employed is the one generated by bacteria. When bacteria on the anode generate current, and protons are released into solution, positively charged species are prevented from leaving the anode by the AEM and therefore negatively charged species move from the middle chamber to the

anode. In the cathodic chamber protons are consumed, resulting in positively charged species moving from the middle chamber to the cathode chamber. This loss of ionic species from the middle chamber results in water desalination without any water pressurization or external electrical energy (Cao et al., 2009).

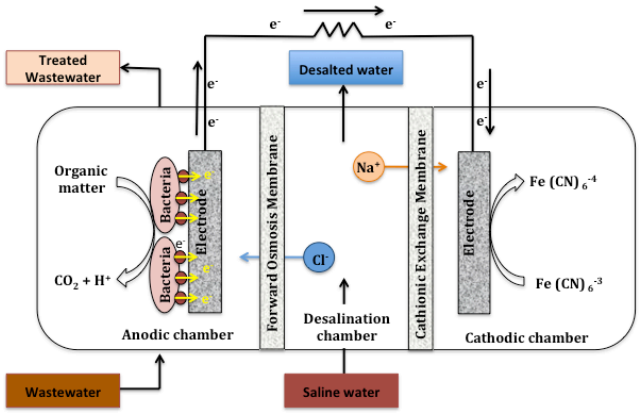

Figure 3. Microbial Desalination Cell diagram

MDC Configurations

The first MDC consisted in a three-chamber device (Cao et al., 2009) that was able to remove the 90 % of salt and to generate a maximum power density of 31 W/m^3 using salt concentrations of 5, 20 and 35 g/l NaCl. The device used acetate as electron donor and ferricyanide as electron acceptor. The same configuration demonstrated to be effective with actual domestic wastewater as anodic substrate (Luo et al., 2012).

The main inconvenient in the MDC operation with a configuration as the one explained above, is the drastic increase of the internal resistance due to the changes in the conductivity of the medium as well as the pH changes in the anodic chamber that entail an impediment for the biofilm growth.

Search for solutions for those situations gave two modifications of the regular configuration of MDC: the **stacked Microbial Desalination Cell** (sMDC) (Chen et al., 2011) that gave higher desalination rates, and the **recirculation Microbial Desalination Cell** (rMDC) (Qu et al., 2012) that allowed a more accurate control of the pH fluctuations.

Regarding sMDC, they achieved an enhancement in the desalination yield by increasing the number of central compartments for desalination (Figure 4) and decreasing the external resistance. This raised efficiency of the MDC performance is because of, per each electron transported through the system, a large number of pair of ions is split (Fan et al., 2008; He et al., 2006).

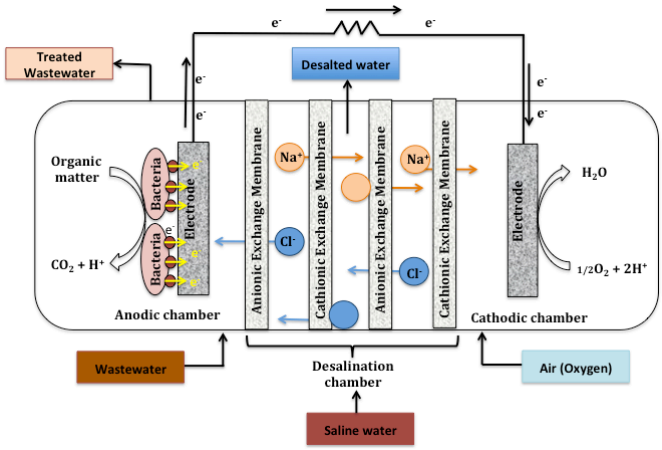

Figure 4. Stacked Microbial Desalination Cell diagram

The results convened that two were the optimal number of desalination compartments with an external resistance of 10Ω. This scheme led to increase desalination rate by 1.4-fold and allowed the substitution of ferricyanide by aired-phosphate buffer which entails not only cost benefits but environmental ones too(Chen et al., 2011).

The second inconvenient in MDC performance is the pH disequilibrium. This pH variation consists in a pH decrease in the anodic chamber due to the protons production of the fuel oxidation meanwhile, in the cathodic chamber the pH increases because of the protons consumption. This disequilibrium entails a shortening in desalination cycles.

There are several steps that have been proposed to solve this pH problem. For instance, to use larger volumes by increasing the frequency of replacement (Cao et al., 2009). Moreover, the addition of acids or bases may also overcome the pH variation (Cao et al., 2009; Chen et al., 2011; Jacobson et al., 2011). In an attempt to eliminate these pH imbalances, rMDC (Figure 5) was designed and operated to allow recirculation of solutions between the anode and cathode chambers using external pumps. In this way, pH variation that could inhibit bacterial metabolism was avoided. The rMDC showed that electrolyte recirculation is an effective method to increase power and achieve efficient desalination (Qu et al., 2012).

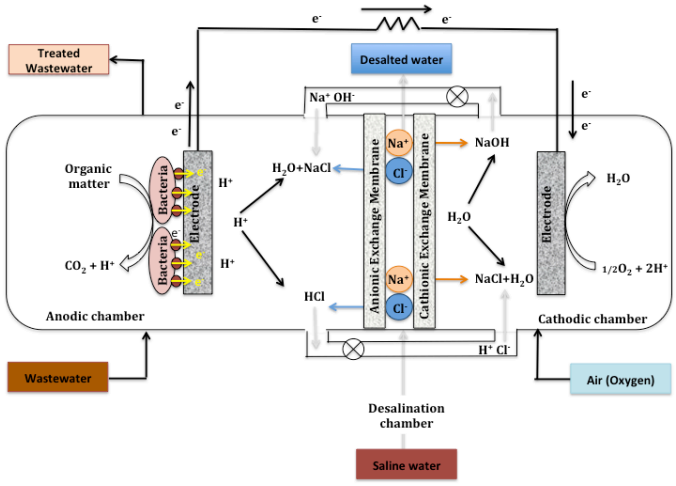

Figure 5. Recirculation Microbial Desalination Cell diagram. Both pumps are represented as the circle:

Due to the results in desalination rate with the sMDC configuration, a combination of MDC with ED design was proposed. The ED is based in a multi-compartments structure with several ionic exchange membranes. This combination led to a MDC with a central desalination compartment formed with a stack of 6 anion exchange membranes (AEM) and 5 cation exchange membranes (CEM). In this way, the system has 10 central serial cells used to circulate the dilute (water to be desalted) in ones and the concentrate (brine, desalted water) in others (Kim and Logan, 2011).

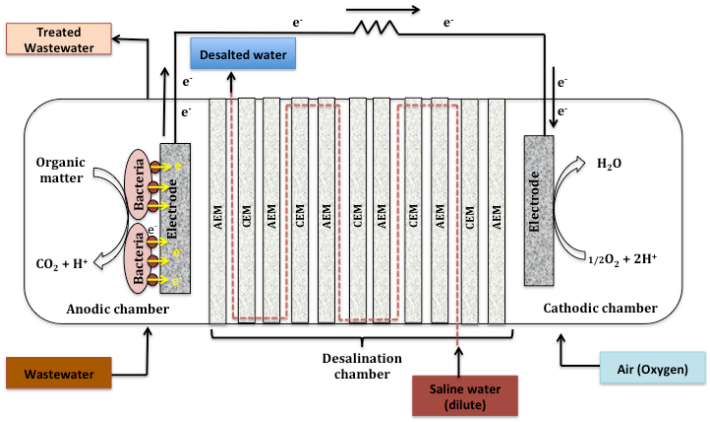

Figure 6. MDC containing an electrodialysis stack as desalination compartment. Red–dots line indicates the dilute flow path with the concentrate flow path counter current to the dilute.

The **ED-MDC** (Figure 6) has reached higher current densities comparable to the MFC ones (Chen et al., 2011). On top of that, the desalination rate has been improved from up to 98%. Furthermore, this configuration has allowed the use of a non-buffered catholyte being no necessary a so strict pH regulation (Kim and Logan, 2011).

In order to improve MDC performance in combination with other already existing membrane technology, a new MDC

configuration was developed. Forward Osmosis (FO) is a desalination technique where there is a water flux between two solutions of different concentration; the feed solution that has a high water potential than the draw solution (Cath et al., 2006; Kim and Logan, 2011; Zhang et al., 2011; Zhao et al., 2012b). In an **Osmotic Microbial Desalination Cell** (Figure 7), the ionic exchange membranes of the MDC are replaced with FO membranes (Cath et al., 2006; Kim and Logan, 2011). In this case, the salts of the middle chamber are not removed but are concentrated since the FO membranes allows not only the water to pass but also reduce the transport of ions to both electrode chambers (Cath et al., 2006; Zhang et al., 2011). Despite FO membranes are more susceptible to fouling, and consequently they increase the internal resistance of the system (Kim and Logan, 2011), it has been reported that fouled FO membranes could increase current generation (Ge and He, 2012). In terms of salt removal, it has been seen that the performance of the device depends on the orientation of the FO membranes. This contradiction in the results entails more studies in this area(Cath et al., 2006).

The substitution of ionic exchange membranes by FO ones obtained better results in MFC than in MDC (Cath et al., 2006; Kim and Logan, 2013; Zhang and He, 2013). Considering this, one innovative method was proposed: the **coupling of Osmotic MFC with conventional MDC** (Cath et al., 2006; Zhang and He, 2012). In this case, the anodic chamber of the Osmotic MFC contains the wastewater while the cathode is fed with saline water. The saline water is diluted due to the water flux from the wastewater of the Osmotic MFC anode. Then, this saline water is fed to the middle chamber of the MDC to finally be desalted. The coupled system

improves overall desalination performance and organic matter removal from wastewater (Cath et al., 2006).

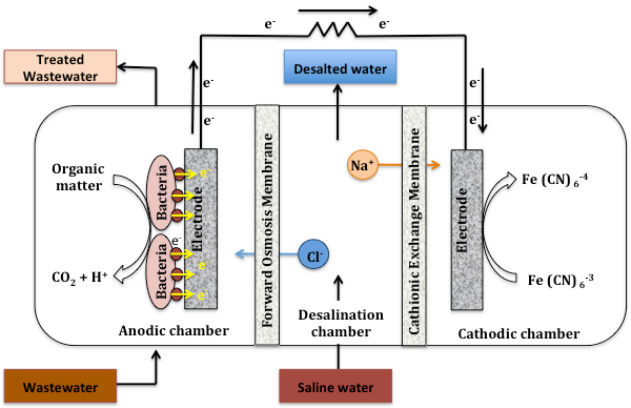

Figure 7. Scheme of an Osmotic MDC. Potassium ferricyanide is usually used as the catalyst in the osmotic MDC, which acts as the electron acceptor in the cathode [64].

In classical MDC, when desalination process is taking place, there is a greatly increase of the ohmic resistance due to the conductivity decrease. This reduction of the salt content limits not only the electricity production but also the desalination rate (Cao et al., 2009). Aiming for the reduction of ohmic resistance in MDC, a new MDC configuration for brackish water and wastewaters was proposed. This configuration consists on the packing of the desalination chamber with anion and cation exchange resins leading to the **ion-exchange resin coupled MDC** (RMDC) (Figure 8).

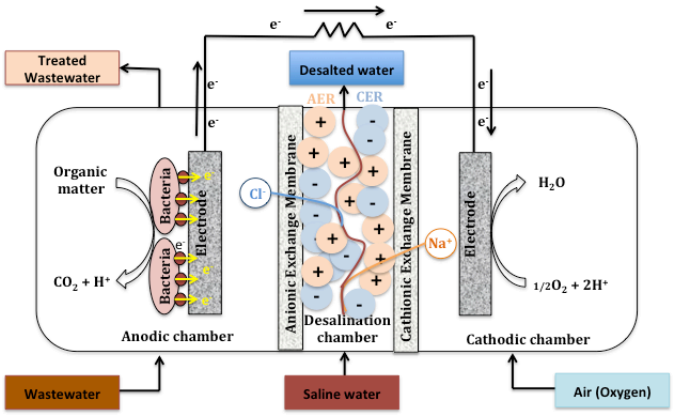

Figure 8. Schematic of ion-exchange resin packed microbial desalination cell (AER: anion-exchange resin; CER: cation-exchange resin).

These resins can operate as ionic conductors when it comes to low salinity water because they are high conductive. Therefore, the stabilization of the ohmic resistance and the reduction of energy consumption in the system are feasible (Morel et al., 2012).

Cathodes in MDC

The first configuration of MDC used ferrycianide as catholyte, although it is a better oxidant than the O_2 in air-cathodes or than Fe^{3+} in graphite cathodes (Cheng and Logan, 2007), it is well known that it is neither sustainable nor cost effective when it comes to environmental costs and real scale applications. Then, air-cathode is proposed as an alternative due to its high reduction potential and cost effectiveness (Bahareh Kokabian, 2013). In fact, high rates of desalination are also achieved with air cathode using carbon cloth electrode with platinum as catalyst (Mehanna et al., 2010). Alternatively, activated carbon can be used to increase the surface area for improve MDC performance.

The disadvantage of use oxygen is the slow kinetics in ambient conditions, requiring more catalytic materials (e.g. platinum) that generally are more expensive. Additionally, high energy is required to maintain a stable concentration of dissolved oxygen in the cathode.

Alternatively, an electrode that uses the microbial community of its surface or in the catholyte as biocatalyst can be used as a cathode. This biocathode catalyse the reduction reaction in the cathodic chamber (Walter et al., 2013). This option in MDC is being successful due to self-regenerating and sustainability (Huang et al., 2011b). The obtaining of high voltages and high desalination rates (Wen et al., 2012), indicated that biocathode could improve the efficiency and performance of MDC. Regarding the microorganisms on cathodes that can accept, directly or indirectly, electrons, there are large variety that can use different TEAs (oxygen, sulphate, nitrate, etc.) (Zaybak et al., 2013) leading to aerobic or anaerobic biocathodes. In aerobic biocathode, MDC operation can be further improved with oxygenic biofilms. They increase the oxygen concentration allowing more electrons to be accepted by the cathode and then, more current flow.

MDC Scale-Up and practical availability

An MDC system can be used either as a pre-treatment method since it can significantly reduce the salt concentration, or it can be used as an individual process for the simultaneously wastewater treatment and desalination.

To date, a MDC system fed by domestic wastewater and that uses the energy produced by itself, has not been implemented at real scale. Although the development of MDC has shown all the advantages of the incorporation of this kind of systems in a wastewater treatment plant, there are still some technical problems not yet resolved that require further research. Problems as the large volume of wastewater needed for the desalination of a small volume of seawater or the acidification or alkalisation of electrode chambers are already issues to solve in terms of scale-up.

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Chapter 4

Test of a Pre-pilot Prototype: Set Up of the Optimal Physiological Conditions

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Strategies for Reducing the Start-up Operation of Microbial Electrochemical Treatments of Urban Wastewater

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ABSTRACT

Microbial electrochemical technologies (METs) constitute the core of a number of emerging technologies with a high potential for treating urban wastewater due to a fascinating reaction mechanism—the electron transfer between bacteria and electrodes to transform metabolism into electrical current. In the current work, we focus on the model electroactive microorganism *Geobacter sulfurreducens* to explore both the design of new start-up procedures and electrochemical operations. Our chemostat-grown plug and play cells, were able to reduce the start-up period by 20-fold while enhancing chemical oxygen demand (COD) removal by more than 6-fold during this period. Moreover, a filter-press based bioreactor was successfully tested for both acetate-supplemented synthetic wastewater and real urban wastewater. This proof-of-concept pre-pilot treatment included a microbial electrolysis cell (MEC) followed in time by a microbial fuel cell (MFC) to finally generate electrical current of ca. 20 Am^2 with a power of 10 Wm^2 while removing 42 g COD day $1m^2$. The effective removal of acetate suggests a potential use of this modular technology for treating acetogenic wastewater where *Geobacter sulfurreducens* outcompetes other organisms.

INTRODUCTION

Urban wastewater treatment is a biological process typically associated with energy consumption due to the air supply required for promoting microbial growth (Paul H Fallgren, 2013). It may be feasible however to turn wastewater treatment into a self-sustaining process by using the energy in the wastewater. The chemical energy contained in the organic matter of wastewater constitutes up to 9-fold more energy than required to treat the wastewater (Heidrich et al., 2011; Logan et al., 2006). Biogas production through anaerobic treatments is the most common technology so far to achieve the goal of self-sufficiency (Muhammad Nasir et al., 2012). However, the use of wastewater as an energy source by using interaction between microbes and electrodes is also feasible (Schröder et al., 2015). These biological redox reactions are at the core of METs (Du et al., 2007; Logan, 2009; 2010; Zhu et al., 2013). From the very beginning of this technology's discovery (Liu et al., 2004) it was proposed to have a promising role in wastewater treatment by allowing for a good effluent quality while converting the biodegradable materials into electric energy.

A large variety of applications have been developed on this basis: direct power generation (microbial fuel cells, MFCs) (Capodaglio et al., 2013; Liu and Logan, 2004; Rabaey and Verstraete, 2005), chemical production of H2 (microbial electrolysis cells, MECs) (Call and Logan, 2008; Logan et al., 2008), microbial electrosynthesis (Rabaey and Rozendal, 2010; Rabaey et al., 2011), water desalination (microbial desalination cells, MDCs) (Cao et al., 2009; Jacobson et al., 2011), or even microbial electroremediating cells (MERCs) for restoring polluted environments (Rodrigo et al., 2014). MFCs and

MECs share a common and similar structure, a community of electroactive microorganisms transferring electrons from organic matter to an electrode (anode) (Wang and Ren, 2013). These electrons are then transferred through an external resistor to a cathode for harvesting electricity (MFC), or to a counter electrode under potentiostatic control (MEC). The presence of these electroactive microorganisms catalyse the oxidation of organic matter on the anode, and donate electrons to the anode that can be harvested as electric current (Logan and Rabaey, 2012). Although urban wastewater has been the most common biodegradable fuel tested in METs, alternative organic matter sources such as cellulose (Rismani-Yazdi et al., 2013), food industry residues (Cercado-Quezada et al., 2010), brewery wastewater (Çetinkaya et al., 2015), cheese wastewater (Kelly and He, 2014), or root exudates (Kouzuma et al., 2014) have been extensively tested in the last decade. The core of a MET-based process mainly lies in: (i) electrochemically active microorganisms (Bond and Lovley, 2003; Jiang et al., 2010; Kim et al., 2004; Richter et al., 2009); (ii) materials for membrane and electrodes (Dewan et al., 2008; Sangeetha and Muthukumar, 2013); and (iii) the operational mode of the system (Erable et al., 2011; Wu et al., 2014). All those factors play an important role in achieving good treatment efficiency and have been greatly studied for increases in operational optimization (Molognoni et al., 2014; Santoro et al., 2014). Since Bond et al. (Bond and Lovley, 2003)[discovered bacteria from the *Geobacter* genus in an electrode-colonizing biofilm, this microbial genus has been the model microorganism for exploring MET. They are not the only microorganism able to colonize an electrode, but they outcompete bacteria from other environments like wastewater (Çetinkaya et al., 2015; Katuri et al., 2012; Lovley et al.,

2011). The reason for that is related to the unique physiology of *Geobacter* to couple its oxidative metabolism with the direct electron transfer to extracellular electron acceptors (Lovley, 2012). A process that is due to *Geobacter's* ability to produce membrane proteins called cytochromes C in large quantities (Mehta et al., 2005). Indeed, those electron carriers are directly involved in the electrochemical activity's mechanism (Busalmen et al., 2008; Esteve-Núñez et al., 2011; Robuschi et al., 2013), a process that fails if a severe reduction of cytochrome C is induced (Estevez-Canales et al., 2015) or it is enhanced under overproduction of key cytochromes (i.e. outer membrane OmcB cytochrome) under sustrate-limiting conditions (Esteve-Núñez et al., 2011; Stephen et al., 2014). Moreover, other novel mechanisms based on microbial nanowires (Malvankar and Lovley, 2014; Malvankar et al., 2015) also participate in the inner biofilm conductivity. The physiological state of electroactive bacteria is key during two different MET operational stages: (i) the start-up period, which is related to the primary colonization and growth of the electroactive biofilm on the electrode surface; and (ii) steady-state period, in which the biofilm is mature and the current harvested by the MET is stable. In this work, we use the model electroactive microorganism *Geobacter sulfurreducens* to explore both, design of new start-up procedures and electrochemical operation where MEC is followed by MFC in order to treat acetogenic wastewater and harvest electric energy. Moreover, the influence of this physiological state is analysed through an ΔOmcB mutant *Geobacter* strain, which confirmed the participation of the key cytochrome OmcB in the achievement of highly-electroactive cells.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

The bacterial strain used was *Geobacter sulfurreducens* DL1. This strain was routinely grown at 30 °C in septum-sealed serum bottles containing freshwater medium (FWM, pH = 6.9, EC = 12.4 $mS \cdot cm^{-1}$) with the following mineral salts: $NaHCO_3$ 2.5 $g \cdot L^{-1}$; NH_4Cl 0.25 $g \cdot L^{-1}$; $NaH_2PO_4H_2O$ 0.06 $g \cdot L^{-1}$; KCl 0.1 $g \cdot L^{-1}$; Fe $(NH_4)_2(SO_4)_26H_2O$ 0.04 $g \cdot L^{-1}$. The medium was supplemented with a trace mineral and vitamin solution (Geelhoed and Stams, 2011). Sodium acetate ($NaC_2H_3O_2$ 20 mM, Sigma-Aldrich, Madrid, Spain) was used as electron donor and fumarate ($C_4H_4O_4$, 40 mM, Sigma-Aldrich, Madrid, Spain) as the sole electron acceptor. The culture media was degassed by using a mixture of N_2/CO_2 (80:20, industrial ALIGAL-12, Air-Liquide, Madrid, Spain). Traces of oxygen were removed from the gas phase by passing the gas mixture through heated copper fillings pre-reduced with H_2. The mutant strain ΔOmcB was obtained from the culture collection of the Microbiology Department of the University of Massachusetts (Amherst, USA). Cultures were grown under same conditions than the wil-type *G.sulfurreducens* but with gentamicin antibiothic in order to assure the knockout phenotype.

In order to obtain *plug and play* cells, a continuos culture or chemostast was used. Chemostat culture consists on an open system where sterile nutrient medium is continuously added to the bioreactor and an equivalent volume of used medium with microorganisms is removed. The physiological steady state is controlled by means of the availability of a limiting substrate. A key parameter that must be fixed in a chemostat is the dilution rate, which is defined as the flow of medium per time over

the volume of culture. At steady state, the specific growth rate of the microorganism is equal to the dilution rate, thus the biomass remains constant (Lee, 2006). The chemostat provides a useful opportunity to study microbial physiology under specific and reproducible conditions, (Esteve-Núñez et al., 2005; Esteve-Núñez et al., 2004).

In our case, the microorganism was grown in a 2 L chemostat (Braun Biostat Bioreactor, Melsungen, Germany). A temperature of 30 °C was kept constant by using a water-jacket device connected to the control unit. Stirring was set at 250 rpm and a level probe was used to maintain the working volume at 2 L. Fresh media water (FMW) for continuous culture experiments was set under fumarate (electron acceptor) limited conditions by using a FWM supplemented with 10 mM acetate and 10 mM fumarate under a growth rate of 0.05 h^{-1}. Both the inlet medium and the bioreactor headspace were made anoxic by using a mixture of N_2/CO_2 (80:20, industrial ALIGAL-12) gas flow. The vessel bioreactor and all associated tubing were sterilized by autoclaving (15 min, 121 °C). Steady-state electroactive cells were obtained after five volume refills. The same set-up and growth protocol were performed in order to obtain steady-state cells of the knock-out ΔOmcB strain.

Microbial Electrolysis Cell Device

A commercial multipurpose Electro MP-1 electrochemical reactor manufactured by ElectroCell (Tarm, Denmark, projected electrode area 100 cm^2) was used as MEC in this work. The cell design (Figure S4) comprised a compact stack design of several polypropylene compartments and neoprene gaskets for an optimal hermetically seal. The arrangement of these compartments allows to

obtaining different configurations from the same cell. In this case, two chambers were used (anodic and cathodic compartments) each with a thickness of 17 mm and a compartment volume 170 cm^3. The anodic compartment was filled with graphite particles (mean diameter 2.3–4.0 mm, porosity 36%). The surface area of the particles was estimated to be 1.173 $m^2 \cdot g^{-1}$. As the total mass of carbon particles in the anode bed was 178 g, the total surface area of the anode bed electrode could be estimated in 208 m^2. However, this anode bed electrode area should be only considered indicative for comparison purpose (for example, with other analogous microbial electrochemical devices using filter press configuration) due to the fact that most of this area is not likely available for biofilm attachment/growth. The cathodic compartment was filled with carbon felt RVG4000 (MERSEN Ltd., Barcelona, Spain). Both compartments contained graphite plates as electrical collectors, while a cationic membrane Nafion 324 (DuPont, DE, USA) separated the compartments. The device was closed with stainless steel screws in order to avoid any leakage of the system. The whole system was completed with a 5 L substrate feed tank and 2 L one for catholyte solution.

As it is shown in Figure S5, the MEC reactor was connected to the anolyte and catholyte tanks by using a Pharmed Tubing 1/4" internal diameter (Saint-Gobain, Courbevoie, France). Two-channel peristaltic pump (205 CA, Watson Marlow, Wilmington, MA, USA) were used to recirculate both streams through the batch mode system with a flow rate of 6 $L \cdot h^{-1}$. Both, the MEC reactor and the anolyte/catholyte tanks were placed in a temperature controlled room at 30 °C. The anolyte tank was kept under anaerobic conditions by fluxing a mixture of N_2/CO_2 (80:20, industrial ALIGAL-12). A reference electrode (Ag/AgCl

KCl 3.5 M) was placed in the geometric centre of the anodic compartment, in order to measure anode potential. A Voltalab PGZ100 potentiostat (Radiometer Analytical, Villeurbanne, France) was connected to the MEC system, allowing recording experimental data while performing to control the electrochemical setup.

Start-up and Operation Procedure

Prior to inoculation, the MEC system was sterilized by recirculation of 70% w/w ethanol/water solution through the whole batch mode system. A filtered gas mixture of N_2/CO_2 was gassed for 2 h in order guarantee both the ethanol evaporation and an anoxic environment inside the MEC.

The anolyte was made of FWM supplemented with 20 mM acetate solution, and the catholyte was made of Na_2SO_4 0.25 M solution. Both solutions were recirculated through the system, and the anode potential (working electrode) was poised at 0.0 V *vs.* reference electrode located in the geometrical centre of the anodic compartment. After overnight operation, the anodic chamber was inoculated by recirculating 200 mL of a batch culture of *Geobacter sulfurreducens* (OD_{600} 0.4) for 2 h in order to ensure the adhesion of cells to the anode particles. In the case of chemostat cells, the anodic chamber was inoculated by recirculating 200 mL of a steady-state culture (OD_{600} 0.11, fumarate concentration <1 mM) for 2 h. Then, the cell cultture solution was replaced by FMW supplemented with 20 mM acetate in absence of other electron acceptor. The MEC was operated without recirculation (pump off) until a positive and measurable current was obtained (3 days for batch cells assay and 12 h for chemostat cells assay). After that, acetate-supplemented FWM was recirculated at a flow rate of 6 $L \cdot h^{-1}$.

Competitive Assays with Soluble Terminal Electron Acceptors

A number of TEAs were tested to evaluate their influence during electric current production in FWM medium. The anolyte tank was pulsed with 1 mL anoxic solutions of the following chemicals: nitrate (1.5 ppm), sulphate (70 ppm), iron (30 ppm and 60 ppm), fumarate (60 ppm and 120 ppm). In addition, oxygen influence was evaluated after air-sparging the anolyte tank until making the solution 2 $mg \cdot L^{-1}$ in oxygen (Oxi 320 Oximeter, Crison, Barcelona, Spain).

Operation as a Microbial Fuel Cell

The main objective of this assay was to demonstrate that the anode biofilm was stable during operation and could achieve high current density. Once the electric current provided by the bioanode reached stable values, it could be assumed that the biofilm had achieved a dynamic balance (equilibrium) between cell division and cell death (or detachment), and thus the system could be considered at steady state, at least from the electrochemical point of view. When the bioanode is stable, the system could be operated as MFC (*i.e.*, device that spontaneously produces electric energy from the oxidation of organic matter in the anodic compartment and reduction of a suitable chemical species in the cathodic compartment, as described before) by disconnecting the potentiostat used for biofilm growth, substituting the catholyte solution with a solution with a chemical substance able to act as electron acceptor, and electrically connecting the anode and cathode collector through and external load (resistor). In this study, a $FeCl_3$ 0.20 M pH = 1 (HCl) was used as catholyte solution (Fe^{3+}/Fe^{2+}, $E° = 0.77$ V *vs.* HSE), a 20 mM acetate + FWM (as described in the previous section) was used as anolyte, and

a 2.1 Ω resistance was used as external load. The volume of anolyte and catholyte was 10 L and 2 L respectively, and the flow rate was 6 $L \cdot h^{-1}$ for both streams (batch operation). Whole configuration represented in Table 1.

	Materials/conditions	Details
BES configuration	Compartments	1 anode compartment; 1 cathode comparment
	Projected area	17 mm
	Compartment thickness	100 cm^2
	Anode electrode	Particles 2.3–4.0 mm diameter mass: 178 g; porosity 36%
	Cathode electrode	Carbon felt
	Electric collectors	Graphite plate
	Membrane	Nafion 324 (DuPont)
	Reference electrodes	Ag/AgCl 3.5 M KCl reference electrodes units located in the geometrical center of each compartment (2 units)
	Flow rate	6.4 $L \cdot h^{-1}$ (both streams)
	Anolyte solution	Acetate 20 mM + FWM (pH = 6.9, EC = 12.4 $mS \cdot cm^{-1}$)
	Catholyte solution	MEC operation: Na_2SO_4 0.25 M (16.0 $mS \cdot cm^{-1}$) MFC operation: $FeCl_3$ 0.20 M pH = 1 HCl (15.6 $mS \cdot cm^{-1}$)
	Anolyte tank	5 L
	Catholyte tank	2 L

Table 1. Bioelectrochemical system (BES) configuration. Freshwater medium: FWM.

The polarization curve was obtained by shifting the value of the connected external load (in the range of 0.25–1000 Ω and then waiting 50–60 min until steady state was reached under a stable electric current generation.

Assays with Real Urban Wastewater

Urban wastewater was harvested from an anaerobic lagoon effluent at the wastewater treatment plant from the municipality of Carrión de los Cespedes (Sevilla, Spain). Physical chemical characteristics are shown in Table 2. The wastewater was filtered under vacuum with a Kitasato flask (Labbox, Barcelona, Spain)

through a Whatman filter paper (Sigma-Aldrich, Madrid, Spain) of 20–25 µm and gassed with a N_2. The urban wastewater was supplemented with acetate to mimic the FWM acetate composition

	Real wastewater				
Parameter	pH	Conductivity ($mS \cdot cm^{-1}$)	BOD ($mg \cdot L^{-1}$)	Total nitrogen ($mg \cdot L^{-1}$)	Acetate (mM)
Value	7.0	1.5	280	0.5	1.0

* Provided by CENTA (Center for New Water Technologies Foundation, Carrión de los Céspedes, Seville, Spain).

(20 mM) so microbial current production could be properly compared. The MEC reactor was run under the same operation conditions used for synthetic FWM assays. The pH buffering assays were performed after bicarbonate addition to the anolyte tank and under N_2/CO_2 (80:20).

Table 2. Wastewater characteristics from the anaerobic lagoon of CENTA treatment plant

Analytical Methods

The content of acetate in the cultures was measured with HPLC with a ZORBAX PL Hi-Plex H Guard Column (50 mm × 7.7 mm, Agilent Technologies, Madrid, Spain) and mobile phase of 0.1% H_3PO_4. The sample volume was 50 µL, mobilized at a flow rate of 0.5 $mL \cdot min^{-1}$. Acetate was detected by using UV at 210 nm. Electric conductivity measurements were carried out using a GLP 31 conductivity meter (Crison, Barcelona, Spain). pH was measured using a GLP 21 pH-meter (Crison, Barcelona, Spain). Both analyses were performed at 25 °C.

Electrochemical Assays

All experiments were performed using a Voltalab PGZ100 potentiostat (Radiometer Analytical, Villeurbanne, France) by the

Voltamaster 4 software. Chronoamperometry was performed at 0.0 V potential *vs.* Ag/AgCl-KCl sat. Reference electrode was located at in the geometric centre of the anodic chamber. Each point was acquired every 10 s. The predictive conversion of acetate into electric current was calculated using the following equation:

$$I = mFn\frac{1}{24\times 3600\times 1000} \quad (1)$$

Where m is the acetate consumption rate ($mmol \cdot d^{-1}$), F is Faraday's constant (96,485 $C \cdot mol^{-1}$), and n is the number of electrons released in the acetate oxidation (n = 8, or eight moles of electrons per mol of acetate oxidized to CO_2).

RESULTS AND DISCUSSION

The bioelectrochemical system (BES) explored in this study aims to oxidize acetate to CO_2 on the anode (*i.e.*, $C_2H_4O_2 + 2H_2O \rightarrow 2CO_2 + 8e^- + 8H^+$, $E°$ = −290 mV). Such a reaction will be tested under two different operational conditions based on the reduction of:

(a) Water on the cathode to produce hydrogen gas (*i.e.*, $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$, $E°$ = −830 mV *vs.* standard hydrogen electrode (SHE) by using a MEC since the reaction is not spontaneous.

(b) Fe^{3+} on the cathode to produce Fe^{2+} through a spontaneous reaction (Fe^{3+}/Fe^{2+}, $E°$ = 0.77 V *vs.* SHE by using an MFC since the reaction is spontaneous.

Microbial Conversion of Acetate into Electrical Current

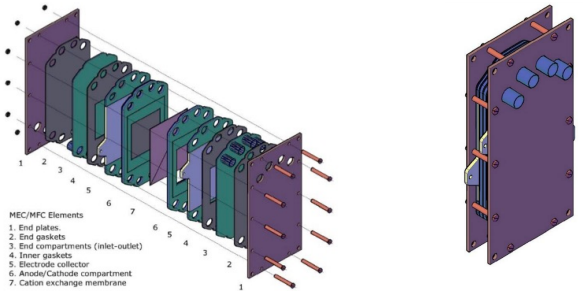
Among the three main operational modes of MET: MFC, microbial short circuited (MSC) and MEC, the last one is the most feasible operational mode for advanced wastewater treatment because of its superior capability for microbial current generation [34]. MEC will be indeed the first operation mode used to test our bioelectrochemical cell.

Our first approach was to use batch-grown cells of *Geobacter sulfurreducens* for inoculating a commercial pre-pilot microbial electrochemical cell reactor (anode set at 0.0 V *vs.* an Ag/AgCl reference electrode).

Under the starting-up operation conditions, batch grown cells showed a long lag period of 10 days before we could notice a current of only 10% of the final steady-state value. In the following week the current density rose continuously until the steady-state value of 1.8 $mA \cdot cm^{-2}$ was reached (Figure 1). During the biodegradation rate that corresponds to a chemical organic demand (COD) removal of *ca.* 6.4 g

COD $day^{-1} \cdot m^{-2}$. It is important to point out that tested conditions were equivalent to a wastewater with high COD soluble fraction and easily degradable organic matter.

a)



(b)

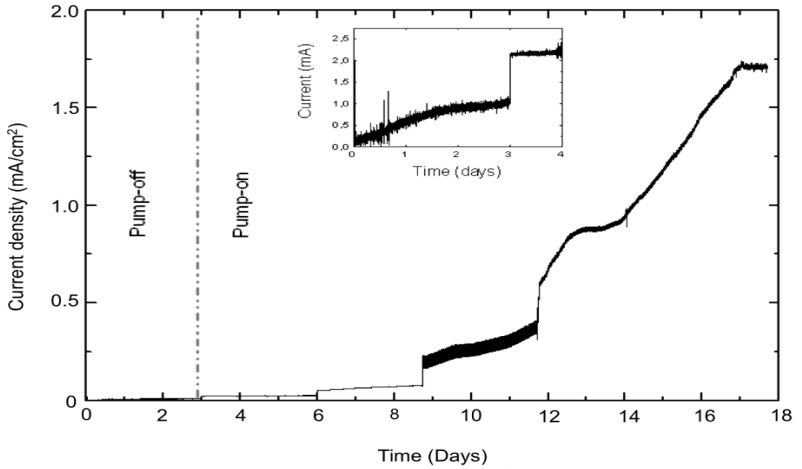


Figure 1. (a) Schematic of the filter press-based bioelectrochemical reactor used for microbial electrolysis cell (MEC) and microbial fuel cell (MFC) operations; (b) electric current production for starting-up the MEC after inoculation with batch-grown cells. Monitoring of current density revealed sudden increases at days 6, 9 and 12. They were due to the replacement the media. Pump-off: peristaltic pump off; Pump-on: peristaltic pump on. Inset: zoom of the electric current production during the first 4 days after inoculation.

Our results (Figure 1) revealed that it was technically possible to operate the MEC at a nominal electric current density of 18 $A \cdot m^{-2}$ (180 mA, 100 cm^2 of cross section). The electric consumption for the system (operating at steady-state) was 61.2 $W \cdot m^{-2}$ (*i.e.*, electric current = 1.8 $mA \cdot cm^{-2}$, cell potential = 3.4 V). Interestingly, part of this energy could be recovered as hydrogen gas (*ca.* 58 $L \cdot day^{-1} \cdot m^{-2}$, 298 K and 1 atm) in the cathode camber although it was not collected in this experimental setup. Furthermore, no biomass presence was found in the anolyte feed tank suggesting that cell growth was restricted the anodic granulated bed.

Minimizing the Start-up Operation: The Use of Plug and Play *Geobacter* Cells

The MEC start-up procedure is key to form an electroactive biofilm on the granulated anode suitable for the oxidation of acetate. Typically the experiments based on *Geobacter* have used batch grown cells at the exponential phase as inoculum (Bond and Lovley, 2003; Geelhoed and Stams, 2011). In contrast, we used an alternative approach based on cells cultured in a chemostat (Esteve-Nunez et al., 2005). Interestingly, it has been previously reported that electron acceptor-limiting conditions in a chemostat enhances extracellular making *Geobacter* highly electroactive even under planktonic conditions (Esteve-Núñez et al., 2011), so we used electroactive planktonic cells for inoculating our MEC system and to analyse the early response as part of the start-up system.

Interestingly, the inoculation of those chemostat-cultured cells drastically reduced the lag-phase of the system so current was harvested from the very beginning (Figure 2). In 12 h the MEC reached 10% of the maximum current, 20-fold faster than using

standard batch cells. During this period acetate was consumed at 6.6 mmol·day^{-1} what correspond to a removal of 42 g COD day^{-1}·m^{-2}. Moreover, a maximal current of 2 mA·cm^{-2} was reached in just 2 days, in contrast with the 17 days required with standard batch-grown cells inoculation performance.

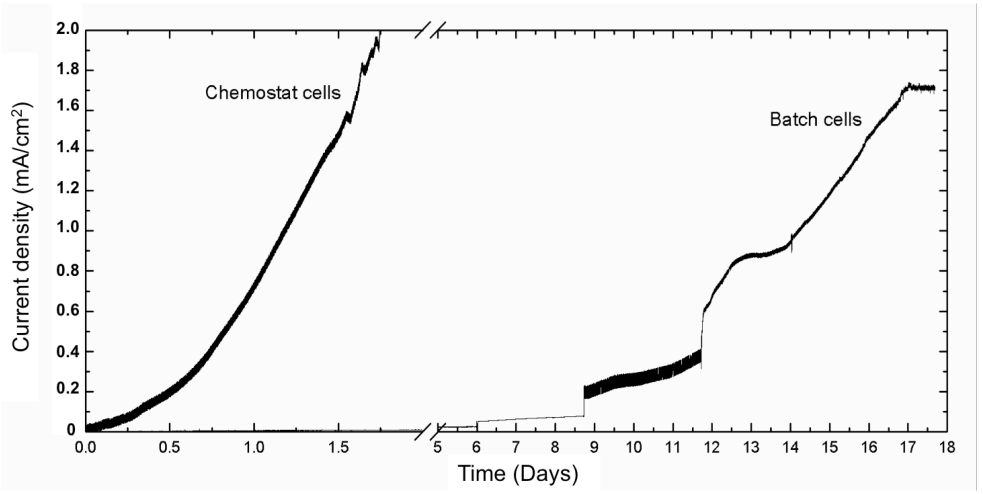


Figure 2. MEC start-up operation using inoculum of *steady-state* cells harvested from a

This result demonstrates that it is possible to start-up a BES in a short period of time when a pre-active inoculum is used to form the biofilm on the anode surface. We have named these cells *plug and play* since they may have an impact on the time required to start-up large microbial electrochemical reactors, one the barriers for the industrial implementations of biofilm-based systems.

Interrogating the Plug and Play *Geobacter* Cells: the use of ΔOmcB mutant strain

It is well known that chemostat provides a great opportunity to study microbial physiology under specific and reproducible conditions, (Esteve-Nunez et al., 2005). In particularly, for *G. sulfurreducens* it has been shown that electron acceptor-limiting condition in a chemostat enhanced extracellular electron transfer rates due to the overproduction of cytochromes at the cell surface (Esteve-Núñez et al., 2011). Transcriptome technique provides valuable information for a better understanding of the metabolic scenario when the cell becomes altered in the ratio electron-donor/electron acceptor. Results of transcriptomic analyses in such limitation conditions demonstrated an up-regulation of several cytochromes, mainly in the outer membrane cytochrome OmcB, which was increased in 3.5-fold (Esteve-Núñez et al., Personal Communication). The key role of OmcB in the extracellular electron transfer to iron oxides was confirmed elsewhere (Leang et al., 2003). In addition, it has been reported that in *G.sulfurreducens* biofilms grown on electrodes, the cells located away from the graphite electrode and hence, in a presumably electron acceptor-limiting conditions (Bonanni et al., 2013; Snider et al., 2012)(Bonanni et al., 2013; Robuschi et al., 2013; Snider et al., 2012), were also enriched in OmcB in a rate of 3.6-fold (Stephen et al., 2014).

Therefore, in order to understand the strategy of the highly electroactive electron acceptor-limiting cells, we electrochemically interrogated a knock-out mutant strain (ΔOmcB) of *G.sulfurreducens* (Figure 3).

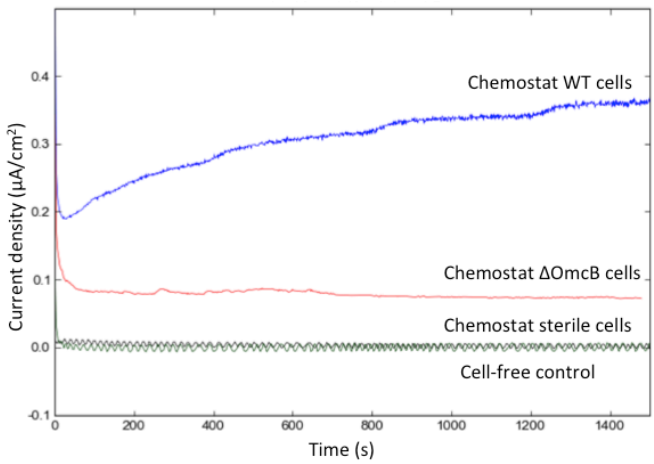


Figure 3. Current production by electron acceptor-limiting wild-type cells (blue) and acceptor-limiting ΔOmcB knock-out cells (red) during DEET process of cells on a graphite electrode. The electrode was initially polarized to 0.2 V and medium contained acetate. Control assay was performed with inactivated cells (black). Bicarbonate buffer was used as cell-free control (green).

Chemostat wild-type cells produced current as soon as they contacted the electrode at a rate almost 3-fold higher than knock-out mutant. Interestingly, the lack of OmcB negatively impact on the electron transfer to the electrode since they reached steady-state at 0.075μA in contrast with the current reached by wild-type cells (0.36 μA). The residual current production in the knock-out strain suggests additional contribution to the EET mechanism that would account for 25% of the current produced by wild-type cells. OmcB is co-expressed with its homologue OmcC, which could partially play the role of OmcB. Besides, recently studies have confirmed the participation of a whole complex cytochrome-porin (in which OmcB is involved) in the electron transfer in the outer membrane (Liu et al., 2014).

These results confirm the key role of OmcB in the high electroactivity shown in chemostat steady-state cells. However, further investigation on additional redox proteins in EET process is required. The transcriptomic analyses of ΔOmcB could give insights into the potential contributors for that 25 % residual current that ΔOmcB strain can produced.

Assay of Potential Electrode Competitors and Inhibitors of *Geobacter sulfurreducens*

When steady state is reached, the electroactive biofilms oxidizes soluble organic matter and use the anode (electrode) as terminal electron acceptor, so it is reasonable to consider that other soluble terminal electron acceptors (TEAs) existing in the aqueous media could affect the MEC operation. They could be an alternative electron acceptor for *Geobacter sulfurreducens* or even they could negatively affect the electroctive biofilm. Nitrate, sulphate, Fe(III) and oxygen are among most common soluble electron scavengers as TEA present in real wastewater. Figure 3 shows the influence on the microbial current production when the anolyte was spiked with different electron acceptor.

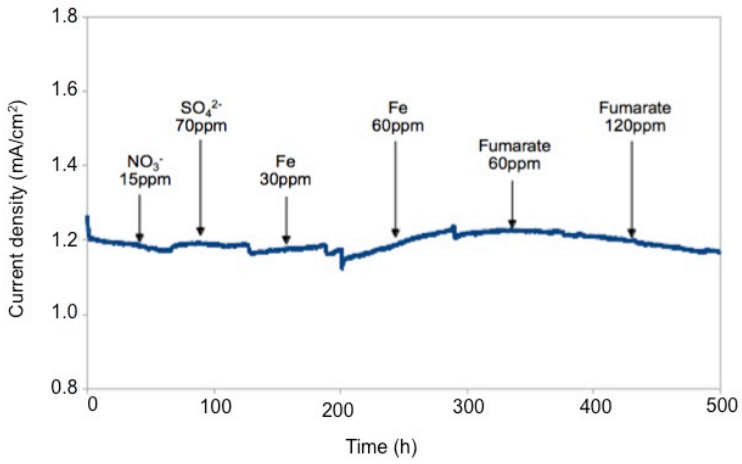


Figure 3. Soluble electron acceptor test on MEC operation behaviour.

Ammonium-oxidizing bacteria present in wastewater are able to generate be up to 5 ppm [52], so the anolyte medium was pulsed with nitrate (15 ppm) although no disturbance was observed on the current production.

In relation to sulphate, moderate concentrations between 20 ppm and 150 ppm can be present in wastewater. Interestingly, the addition of 70 ppm of Na_2SO_4 to our inlet media did not show any effect in the current production.

Iron is normally present in relatively low concentrations (less than a few ppm) due to its low solubility at neutral pH and it is typically associated to industrial wastewater discharges. In our case, iron was added to the system in the form of Fe (III)-citrate salt at two different concentrations, 30 ppm and 60 ppm. This concentration was quite high compared with the typical natural level of Fe (III) in domestic wastewaters (less than 3 ppm), but taking the high affinity between iron and *Geobacter* into account (Mahadevan et al., 2006) we considered testing its competitive role under high doses.

On the other hand, some other soluble compounds produced in the degradation of organic matter could be used as electron acceptors. Thus, we tested fumarate, a known TEA, that is reduced to succinate (fumarate + $2H^+$ + $2e^-$ → succinate, $E°$ = + 0.031 V) by *Geobacter sulfurreducens* as part of its respiratory system. As in the previous assays, no current alteration was detected after the TEA Finally, the competitive effect of oxygen was also tested. Although *Geobacter sulfurreducens* is often wrongly classified as a strict anaerobe, it has been reported to respire oxygen when supplied at low concentrations (10%) (Lin et al., 2004), so oxygen could act as a true electrode competitor for accepting electrons from microbial metabolism. In addition to this physiological role, oxygen may also be toxic over certain levels by oxidizing the cytochrome network and blocking the electron transfer. Furthermore the presence of oxygen can generating reactive oxygen species (hydrogen peroxide, superoxide radicals), which can damage cell structure, including membrane, DNA and proteins, in a process, referred as oxidative stress (Nunez et al., 2004). Indeed a current drop was measured in our MEC device when oxygen level was increased to 2 ppm (Figure S1). In spite of this negative response, the electroactive biofilm was robust enough to recover the value of steady-state current after restoring the anoxic conditions of the inlet medium.

Microbial Electrolysis Cells Performance with Real Wastewater

On top of the TEAs, real urban wastewater contains undetermined amounts of diverse recalcitrant compounds like drugs and personal-care chemicals (Nakada et al., 2007) together with trace pollutants like heavy metals (Cr^{4+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) (Fu and

Wang, 2011) that could be potentially harmful to the biofilm cells, so a real urban wastewater sample was used as matrix for the preparation of the inlet medium in order to monitor the MEC performance. In addition to the COD content of the wastewater, additional acetate was supplied in order to assure a suitable electron donor for *Geobacter sulfurreducens*. Interestingly, the stationary state current was increased from 20 mA to 37 mA when the acetate-depleted synthetic inlet media was shifted to an acetate-supplemented urban wastewater media. In spite of the positive using a synthetic freshwater medium (150–175 mA). Such a difference could be due to the non pH-buffered nature of the wastewater if we consider the critical role of biofilm acidification due to acetate oxidation in electroactive *Geobacter sulfurreducens* biofilms (Torres et al., 2008).

To counterbalance the acidification of the biofilm we proceeded to add bicarbonate, the standard buffer salt used as growth media in *Geobacter* (Geelhoed and Stams, 2011). Interestingly, sequential additions of bicarbonate generate a steady-state current of 140 mA (Figure 4). This suggests that the dynamic balance between proton formation inside the biofilm and the buffer capacity of the bicarbonate in the bulk solution have an impact on the electroactive biofilm's performance (Torres et al., 2008). Apparently, acetate oxidation coupled to electrode reduction produces more protons than those neutralised by bicarbonate diffusing from bulk solution to the biofilm. Then, acetate oxidation and subsequently current production (*i.e.*, extracellular respiration rate) will be limited to avoid further damage to the biofilm. Furthermore, the electric conductivity shift provided by the addition of HCO_3^- ions to wastewater was not considered responsible of the current enhancement, since the

electrical conductivity (12 $mS \cdot cm^{-1}$ at 25 °C) was similar to the one present in the synthetic medium.

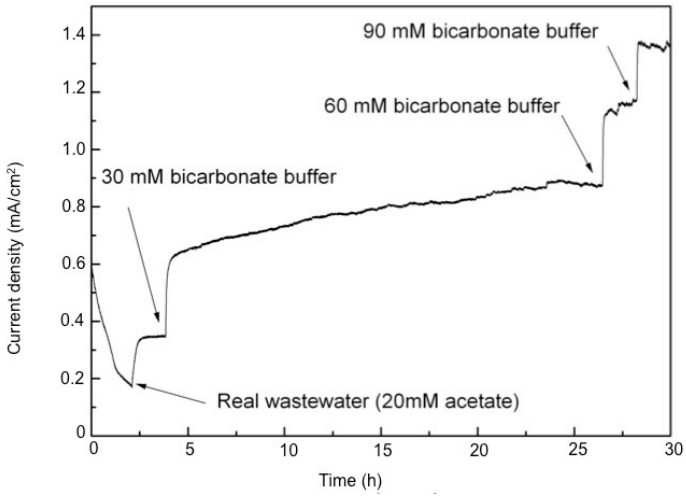


Figure 4. Electric current measured in MEC system when real water matrix is used as fuel (anode at 0.0 V *vs.* a 3.5 M Ag/AgCl reference electrode). Water matrix is supplemented with 20 mM acetate (see Section 2.5).

Microbial Fuel Cell: Steady State Operation and Power Curve

As described previously, once the steady state has been reached in a MEC (*i.e.*, the electric current is stable), the reactor was converted into a MFC by shifting the catholyte solution to $FeCl_3$ 0.2 M, pH = 1 HCl, then connecting anode and cathode through an external load (resistor 2.1 Ω (Figure S2).

The anode potential value was in the range of −150 mV and −350 mV (*vs.* an Ag/AgCl reference electrode), indicating that the oxidation of acetate to produce CO_2 was occurring on the anode. Consequently, the reduction of Fe^{3+} produced on the cathode was at a potential of between 350 mV and 450 mV. The slight change of the cathode potential during the experimental period was mainly due to the change of the formal potential ($E = E° + RT/nF\ln[Fe^{2+}]/[Fe^{3+}]$) as Fe^{3+} concentration decreased and Fe^{+2} concentration increased during the experiment. The membrane potential drop is considered as an indirect value of the internal resistance of the MFC. In our system it was in the range of 250–400 mV, indicating that an important amount of available energy produced by the oxidation/reduction reaction (*i.e.*, $E°_{cell} = E°_{cathode} - E°_{anode} = 0.77\ V - (-0.29\ V) = 1.06\ V$ in standard conditions) was consumed inside the cell as ohmic drop, lowering the energy efficiency of the system. On the other hand, the measured variations of the anode potential and the membrane potential drop could be attributed to the variations in the electric field inside the cell due to liquid/particles movements and turbulences created inside the anode compartment in the cell.

Figure S3 shows a current density for the MFC in the range of 1.75–2.25 $mA{\cdot}cm^{-2}$ (current intensity 175–225 mA). As previously described, the slight decrease is due to the change in the cathode equilibrium potential that affects current density production.

As the cell's potential depends on the anode's and cathode's potential, as well as the membrane potential drop (*i.e.*, $E_{cell} = E_{cathode} - E_{anode} - E_{mem\_drop}$, being $E_{mem\_drop} = I\ (R_{membrane} + R_{anode\_compartment} + R_{cathode\_compartment})$, it is important to study each parameter separately in order to understand the MFC operation. Figure 5 shows the anode, cathode and membrane potential drop *vs.* electric current density for the experiment studied in this section (performed as described in the Materials and Methods section). It is important to note that the slopes for both the anodic and cathodic processes (overvoltage) were higher (absolute value) in the case of bioanode potential and closely related to kinetic hindrance considerations. It can be assumed that the cathode reaction was faster than the anode reaction (catalyzed by the microorganism biofilm), and that explains the different value for the slopes in both processes. Another important point is the membrane potential drop (green line in Figure 5), it represents an important energy loss inside the system. The high value of the membrane potential (300 mV at 1.75 $mA \cdot cm^{-2}$) could be related to the low electrical conductivity of the anode solution. Indeed, in this case the main limitation of the system was the high value (*i.e.*, slope) of the membrane potential drop. In a real application the electrical conductivity of the anode stream is determined by the dissolved salts, which is a difficult parameter to change. Reducing the thickness of the anode compartment may be a strategy to optimize the system performance in order to reduce the ohmic drop. Moreover, the use of carbon particles with different size or diameter in order to increase the bed´s porosity (*i.e.*, surface area available for the biofilm) could be a suitable strategy for optimization.

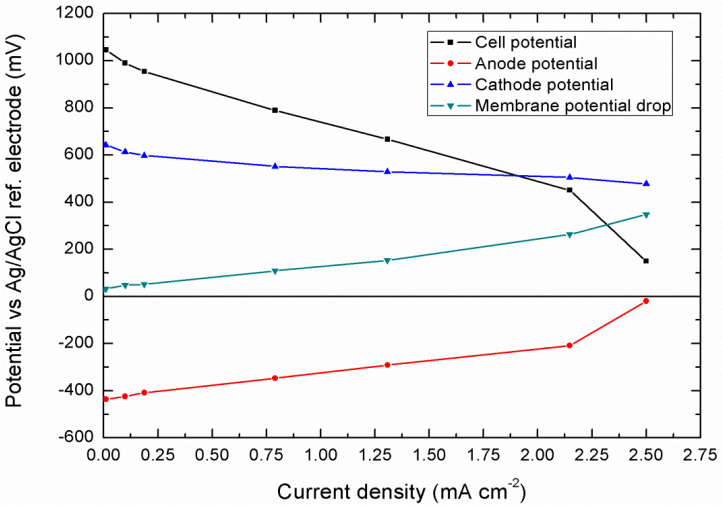


Figure 5. Potential diagram for the MFC device at steady state.

When the MFC was operated at short circuit (external load = 0 Ω) the electric power provided by the device was zero, but the consumption of acetate (*i.e.*, wastewater treatment rate in a real application) was maximal. Alternatively, if the MFC is operated at (or near) open voltage circuit conditions (for example, external load = 10 kΩ), the MFC provides the maximum cell voltage, but the wastewater biodegradation rate would become almost zero. Figure 6 shows the cell potential and the electric power provided by the cell *vs.* current density. The maximum power was reached at 2 $mA \cdot cm^{-2}$ when the cell voltage was 500 mV and the electric power density provided by the MFC was 10.0 $W \cdot m^{-2}$. This operational condition was reached when an external resistor of 2.1 Ω was connected as external load.

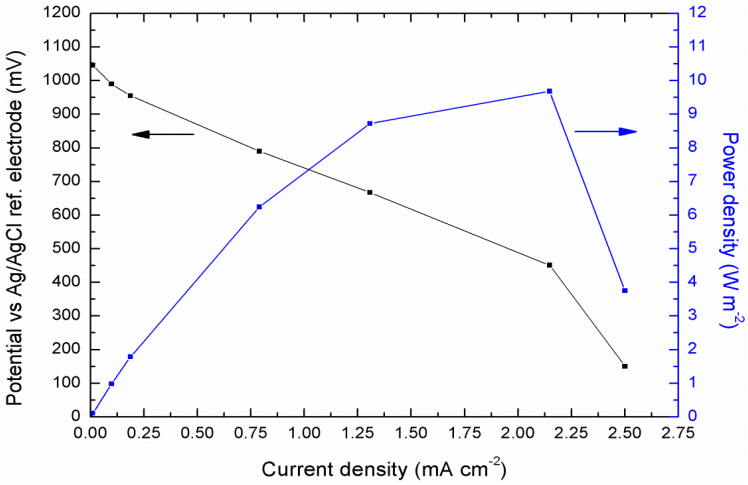


Figure 6. Cell potential and electric power density provided by the MFC at steady state (batch operation mode).

CONCLUSIONS

BESs are suitable technologies for treating urban wastewater, however a number of factors should be explored in order to optimize the methodology and make them profitable. Although materials like ion interchange membranes and electrodes are typically the key issues under investigation, we have tried a different strategy based on exploring the physiology of the inoculum under a fine-tuning method to optimize the start-up operation. Our chemostat-grown cells were able to reduce the start-up period by 20-fold while enhancing the COD removal by more than 6-fold during the start-up period. We demonstrated, through the electrochemical assays with the ΔOmcB mutant strain, the key role of OmcB cytochrome in the highly efficient phenotype of the chemostat-grown cells. This phenotype led to an electroactive biofilm robust enough to the inhibitory action of additional electron acceptors present in the wastewater. However, the pH of the medium was key for harvesting maximal current. The methodology described in this paper has been successfully tested for both acetate-supplemented synthetic wastewater and real wastewater as a proof-of-concept for a pre-pilot treatment where MEC was followed by a MFC. The effective removal of acetate suggests a potential use of the technology for treating acetogenic wastewater from an anaerobic digester reducing the start-up operation by using plug and play *Geobacter* cells. The modular nature of our system allows a feasible scale-up of the technology.

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SUPPLEMENTARY INFORMATION

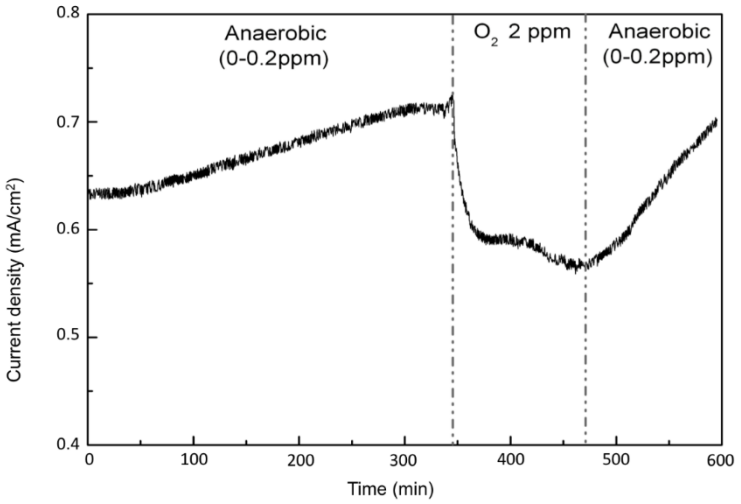


Figure S1. Electric current measured in microbial electrolysis cell (MEC) operation mode (anode at 0.0 V *vs.* reference electrode Ag/AgCl 3.5 M) under different oxygen levels

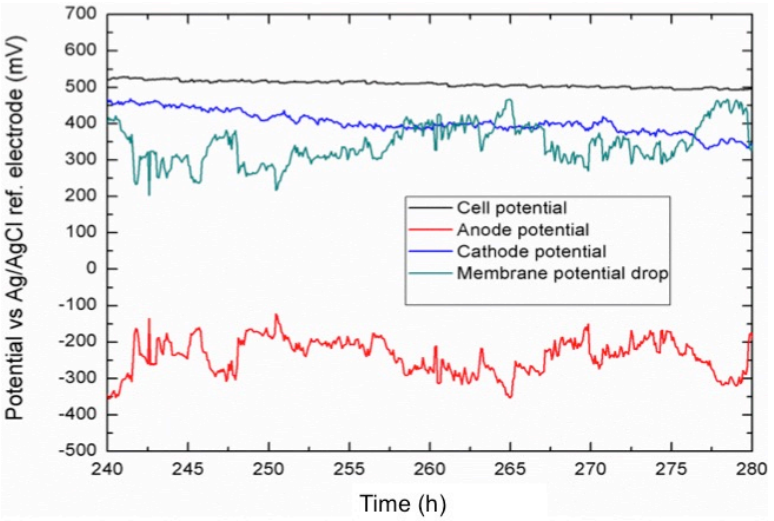


Figure S2. Anode, cathode, cell potential and membrane potential drop for the microbial fuel cell operation mode. Anolyte 20 mM actetae + freshwater medium (FWM). Catholyte FeCl3 0.2 M pH = 1 (HCl). External resistor: 2.1 Ω. Flow rate: 6 $L \cdot h^{-1}$. Bath operation mode.

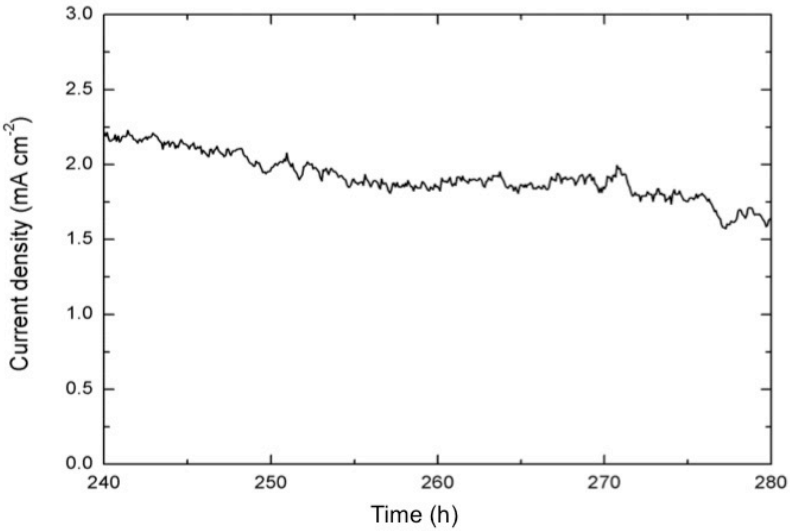


Figure S3. Current density during the microbial fuel cell (MFC) operation mode.

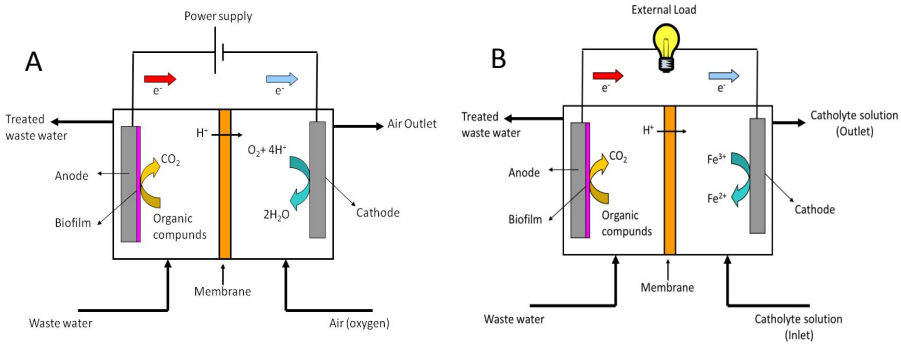


Figure S4. Diagram of the MEC (A) and MFC (B) configuration.

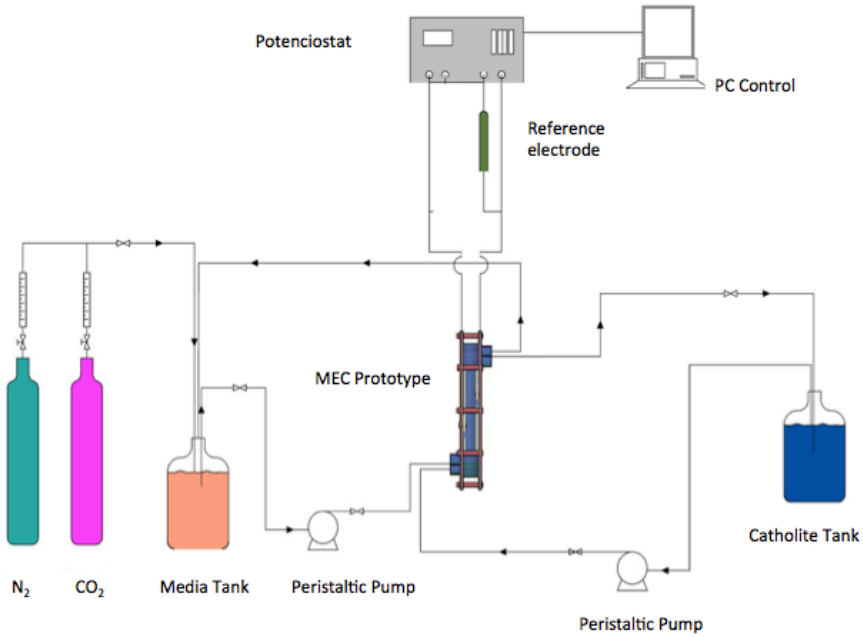


Figure S5. Flow diagram of the experimental bioelectrochemical system (BES).

Chapter 5. Desalination with a pre-pilot MDC system with pure culture of *Geobacter sulfurreducens*

Submitted to *Applied Energy*

Strategies for merging Microbial Fuel Cell technologies with water desalination processes

Zulema Borjas, J.M. Ortiz and Abraham Esteve-Núñez

ABSTRACT

Microbial Desalination Cells constitutes an innovative technology where microbial fuel cell and electrodialysis are merged into a device for producing fresh water from saline water with no energy-associated costs. Acetate in acetogenic wastewater was the organic fuel for the system. Acetate was converted into electrical current (j=0.08 mA cm^{-2}) by an anodic biofilm of the electroactive bacteria *Geobacter sulfurreducens*. In this work, we produced a system with an efficient start-up protocol where desalination up to 87 % occurred in a 3 days cycle using a filter press-based MDC prototype without any energy supplied for the desalinization process. This protocol is optimized for start-up time with a simplified operation procedure making it a more feasible strategy for scaling-up either as a single process or as a pre-treatment method for other well established desalination technologies such as reverse osmosis (RO) or reverse electrodialysis.

INTRODUCTION

Shortages of fresh water is a major challenge for societies all over the world. In this sense, current water desalination technologies can significantly increase the water resources for drinking, industrial use and irrigation, but require significant electrical or thermal energy. On top of the environmental impact due to brine emissions (Elimelech and Phillip, 2011), reverse Osmosis (RO) consumes at least 3 $kWhm^{-3}$ of electric energy (MacHarg et al., 2008), while thermal technologies can reach more than 7 $kWhm^{-3}$ to drive desalination processes. High-energy costs are the main drawback of membrane-based desalination technologies so new technological options are needed to generate desalinated water at a reasonable price.

Microbial Electrochemical Technologies (METs) constitute a platform (Schröder et al., 2015) of emerging technologies with a high potential for energy production coupled to wastewater treatment. These technologies are based on the interaction between electroactive bacteria and electrodes, allowing the direct transformation of soluble organic matter into electrical current (Schröder et al., 2015) this variant of METs is called the microbial fuel cell (MFC).

In the same context of using microorganisms for harvesting energy, the Microbial Desalination Cell (MDC) represents another promising technology for low cost desalination and simultaneous wastewater treatment. MDC could be considered as the integration of MFC and an electrodialysis (ED) cell in order to achieve the objective of treating wastewater, desalinating water, and producing clean and renewable energy in one step. (Cao et al., 2009a). Indeed, the versatile and simultaneous applications of MDC have made of it a real and

feasible alternative for both desalination and wastewater treatment (Kim and Logan, 2013a, 2013b).

The MDC unit is composed by at least three chambers (Figure 1): 1) an anaerobic anodic chamber that contains the electroactive microbial community which oxidises the organic matter contained in wastewater and then transfers electrons to the anode; 2) a central desalination compartment separated from the others by an anion exchange membrane (AEM) and a cation exchange membrane (CEM); 3) a cathodic chamber where the reduction counter-reaction occurrs. In a MDC reactor, cation and anion-exchange membranes are alternatively placed between the cathodic and the anodic compartment, as indicated in Figure 1. Moreover, an external load is placed between anode and cathode collectors, allowing the flow of electrical current.

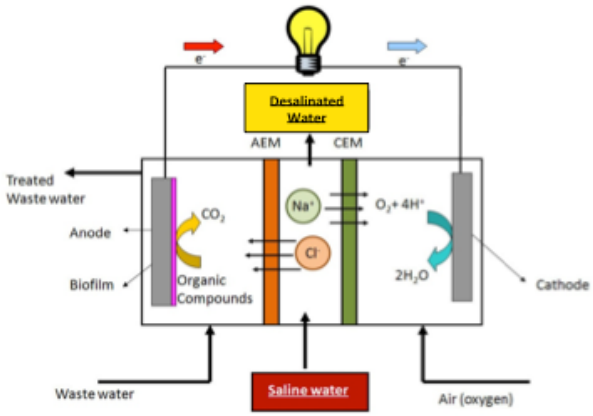


Figure 1. Diagram of MDC unit

If organic matter is used to feed the anodic compartment, and the cathodic compartment is fed with a catholyte (for example, Fe^{3+} or

oxygen in acidic solution), then a potential difference is established between the electrodes. Eventually, anions and cations migrate through their respective membranes raising the salt concentration in the anodic and cathodic compartments while decreasing it in the saline compartment (central compartment). Interestingly, the MDC constitutes a sustainable process since it does not require external energy input because microbes harvest it from waste.

Different MDC designs have been reported in the literature from the first proof-of-concept study (Cao et al., 2009b), including stacked MDCs (Chen et al., 2011), biocathode MDCs (Wen et al., 2012) and recirculation MDCs (Qu et al., 2012).

Regarding the efficiency of the desalination process, the main limitation in the MDC are i) the drastic increase of the internal resistance due to the changes in the conductivity of the medium, and ii) the pH variations in the anodic chamber that can affect the biofilm growth. Additionally, most of the studies on MDC operation follow the same start-up protocol: a pre-growth of the biofilm on the anode under a MFC configuration (Cao et al., 2009b; Kim and Logan, 2013b), and then a conversion of MFC into a MDC device by adding a supplementary chamber (saline compartment) that requires the disassembly of the whole system. This strategy for starting-up MDC is a time-consuming procedure that can require almost 20 days (Meng et al., 2014) and does not favour reproducible experimental results at the lab scale. Furthermore, most of the previous studies were conducted on millilitre-scale MDCs (<300 ml) where, to the best of our knowledge, few studies reported MDC at the scale of 1 liter of volume (Jacobson et al., 2011; Zuo et al., 2014). Such limitations in volume prevent the understanding necessary for MDC scaling up into pre-industrial prototypes.

In this work, we have overcome some the previous limitations by using an efficient start-up protocol where desalination of up to 94 % occurs from the first cycle with the final structural configuration. The MDC operates autonomously without any additional energy supply for desalination and can be used for the systematic study of lab scale MDC performance under different experimental conditions.

EXPERIMENTAL SECTION

Bacterial strain, culture condition

A pure and continuous culture of *Geobacter sulfurreducens* strain DL1 was used as inoculum for the MDC start-up protocol. The continuous cells were obtained in 2 L chemostat (Braun Biostat Bioreactor, Melsungen, Germany). A temperature of 30 °C was kept constant by using a water-jacket device connected to the control unit. Stirring was set at 250 rpm and a level probe was used to maintain the working volume at 2 L. Fresh media water (FMW) under fumarate (electron acceptor) limited conditions (10 mM acetate and 10 mM fumarate) (Esteve-Nunez et al., 2005) was fed into the reactor with a dilution rate of 0.05 h^{-1}. Both the inlet medium and the bioreactor headspace were made anoxic by using a mixture of N_2/CO_2 (80:20, industrial ALIGAL-12) gas flow. The vessel bioreactor and all associated tubing were first sterilized by autoclaving (15 min, 121 °C). Steady-state electroactive cells were obtained after five volume refills.

Microbial Desalination Cell device (MDC)

A commercial multipurpose electrochemical reactor manufactured by ElectroCELL company (Electro MP-1, projected electrode area 100 cm^2) was used as the MDC device. The MDC

prototype (Figure 2A) had a compact stack design consisting of several polypropylene compartments and neoprene gaskets for a hermetic seal. The dynamic design of the device allows different cell configurations. In this case, a three compartments configuration was used. The desalination chamber (compartment volume: 70 cm^3) separated the anodic and cathodic chambers (compartment volume of each: 70 cm^3) by an anionic exchange membrane (ACS Neosepta) and cation exchange membrane (CMX Neosepta), respectively. Both electrodes (i.e. anode and cathode), were composed of carbon felt RVG 4000 (MERSEN Ltd.) and contained graphite plates as electrical collectors.

The device was closed with stainless steel screws in order to avoid any leakage. The whole system was fed by three tanks of 2 L volume for anolyte, catholyte and saline solutions. Anolyte solution consisted of FWM supplemented with 20 mM acetate as sole electron donor in both operation modes, MEC (with power supply) and MDC (no power supply). The catholyte and saline solution were different depending on the operation mode: in MEC, the catholyte was a solution of 0.025 M $NaSO_4$ while the saline solution was 30 mM of $NaHCO_3$; under MDC conditions, the catholyte was 3 % NaClO (pH 12) and the saline solution was 86 mM (5 g/l) NaCl. The volume of the saline solution was dependent on each experiment performance (2 L in MEC, 0.2 L in MDC).

Pharmed Tubing with an internal diameter of 6.35 mm (SAINT-GOBAIN) connected the three tanks to the MDC prototype. A four-channel peristaltic pump (Heidolph Pumpdrive 5201) was used for recirculation in the system. Figure 2B shows a flow diagram of the MDC experimental set-up. The flow rate of both solutions was of 75 mL min^{-1}. The whole system was operated in a temperature-

controlled room at 30 ºC and kept under anaerobic conditions by flushing a mixture of N_2/CO_2 (80:20, industrial ALIGAL-12) into the tanks. Two reference electrodes (Ag/AgCl KCl 3.5 M, CRISON) were placed into the cell, one in the geometric center of the anodic compartment and the other one in the cathodic compartment, in order to measure anode and cathode potential respectively. A power supply (Aim-TTi, 0-15 V, 0-5 A) was connected to the electrochemical reactor to be used during the start-up protocol.

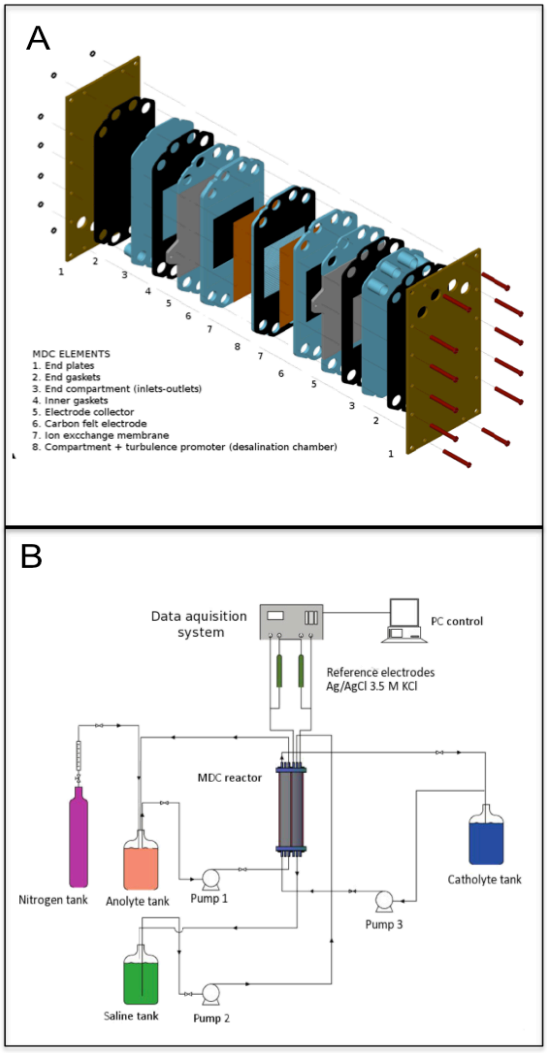


Figure 2A) MDC configuration; 2B) Flow diagram of MDC experimental set-up.

Start-up and operation procedure

MDC configuration testing

Preliminarily conventional electrodialysis under abiotic conditions was performed in order to test if our prototype configuration was feasible for desalination. A 2 L solution of FWM was used as anolyte and a 0.025 M Na_2SO_4 solution as catholyte. A power supply was employed to apply a cell potential of 3 V between anode (positive terminal) and cathode (negative terminal). The saline stream consisted on 2 L of 30 mM $NaHCO_3$. The system was operated in batch mode with recirculation by peristaltic pump.

Start-up procedure

Prior inoculation, the cell was sterilized by circulation of 70 % V/V ethanol/water solution through the whole system (prototype and tubing). A filtered gas mixture of N_2/CO_2 was passed through the MDC for 2 hours to guarantee ethanol evaporation and an anoxic environment inside the device. Also, electrolytes and saline solution were degassed. The anolyte solution was 2 L of FWM supplemented with 20 mM acetate (without any terminal electron acceptor, pH 6.95, CE 5.95 mS cm^{-1}), the catholyte solution was made of 2 L of 0.025 M Na_2SO_4 (pH 7.87, EC 4.74 mS cm^{-1}) and the saline solution was 2 L of 30mM sodium bicarbonate (pH 8.70, EC 5.1 mS cm^{-1}). The volume relation of the electrolytes and saline solution was of 1:1:1 ($V_{an:}$:V_{cat} : V_{desal}). The cell potential was fixed at 1.0 V (i.e. potential between anode and cathode) and all three solutions were recirculated through the system for 2 hours prior to anode inoculation in order to assure no dead volumes or stagnant zones throughout the system. After this, pump recirculation was switched off. Then, the anodic chamber was

inoculated with 300 ml of chemostat *Geobacter sulfurreducens* culture. After inoculation, the whole system remained without recirculation overnight to ensure cell adhesion to the anode. Then, recirculation was continued with the first desalination cycle as part of the start-up protocol. This involved sodium bicarbonate desalination in the MEC configuration with a voltage of 1.0-1.5 V being applied. pH and electric conductivity (EC) measurements were carried out periodically during the start-up stage.

Desalination operation

Once the start-up stage was performed (i.e. electric current plateau was obtained), NaCl desalination was carried out by switching to MDC mode. The peristaltic pump and power supply was turned off and disconnected from the electrodes. Then, MDC electrode collectors were connected to an external load of 2.5 Ohms. The value of the external load selected was to ensure proper measurement of the electric current in the system by voltage drop measurement. Anolyte was replaced with a fresh solution (same composition and same volume, see above) while the catholyte was replaced with a 3 % NaClO 2 L solution (pH 11.14, EC 14.57 mS cm^{-1}). The saline solution was replaced with 0.2 L of 86 mM NaCl. The volume relation was 10:10:1 ($V_{an:}$:V_{cat} : V_{desal}). Once all the tanks were replaced, recirculation pump was switched on, and desalination was started. Measurements of pH and electric conductivity (EC) of the solutions was taken periodically during each desalination cycle, anolyte samples were taken for Chemical Oxygen Demand (COD) analysis.

Electrochemical equipment

The data acquisition for the anode/cathode potentials and

electric current was performed using a custom Visual Basic Program and ModBus modules (ICP-DAS). Reference electrodes (Ag/AgCl 3.5 M, CRISON) were placed in the anode and cathode compartments, using lugging capillary (Teflon).

Analytical methods

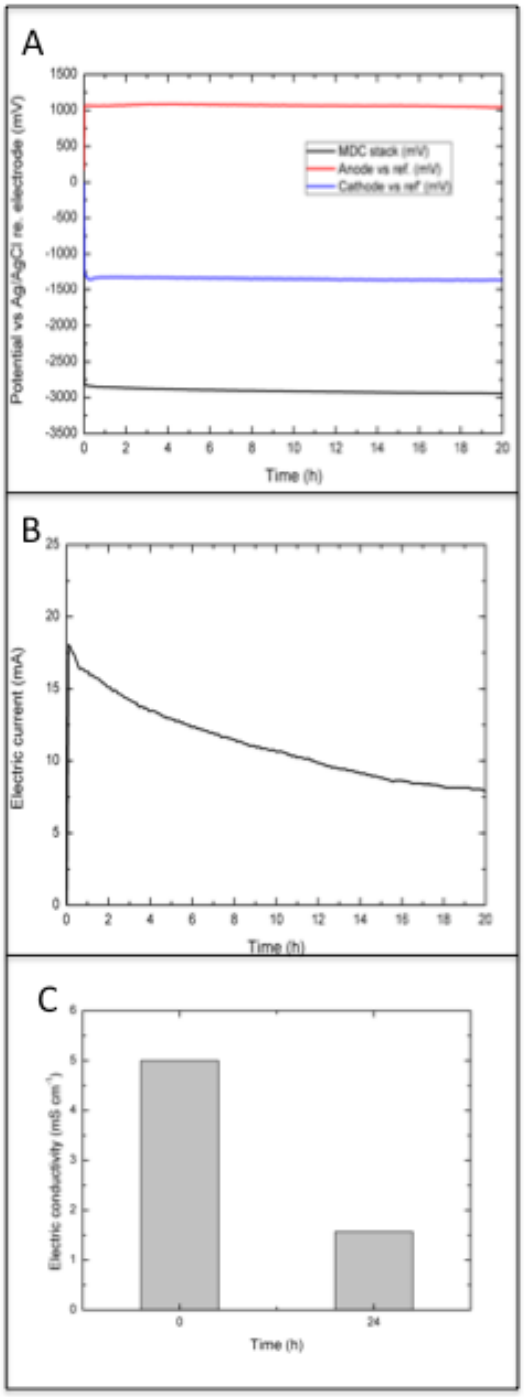
Electrical conductivity measurements were carried out using GLP 31 conductivity meter (CRISON). pH was measured using a GLP 21 pH-meter (CRISON). Both analyses were performed at 25 ºC. For determining total COD, 15 mL of sample were collected and kept at 4ºC until analysis by APHA method 5520.

The content of acetate in the anolyte was measured with HPLC with a ZORBAX PL Hi-Plex H Guard Column (50 mm × 7.7 mm, Agilent Technologies, Madrid, Spain) and a mobile phase of 0.1% H_3PO_4. The sample volume was 50 µL, mobilized at a flow rate of 0.5 $mL \cdot min^{-1}$. Acetate was detected by using UV at 210 nm.

RESULTS AND DISCUSSION

Testing desalination under an abiotic MDC configuration

In order to characterize the performance of the cell stack under non-limiting conditions abiotic electrochemical desalination was carried out. An electric potential of 3.0 V was applied between anode (positive terminal) and cathode (negative terminal). Under this condition, water was oxidised on the anode ($2H_2O$ (l) $\rightarrow O_2$ (g) + $4H^+$ + $4e^-$, E^0=-1.23 V) while water was reduced on the cathode ($4H_2O$ (l) + $4e^- \rightarrow 2H_2$ (g) + $4OH^-$, E^0=-0.42 V) and a solution of 0.025 M $NaHCO_3$ was desalinized in the middle compartment (Figure 3C).



The electrode's potential remained constant throughout the desalinization process (Fig 4A). It is known that the potential of the whole MDC should correspond to the following equation:

$$E_{cell}=E_{cathode} + E_{anode} + R_{cell}\, I_{cell}$$

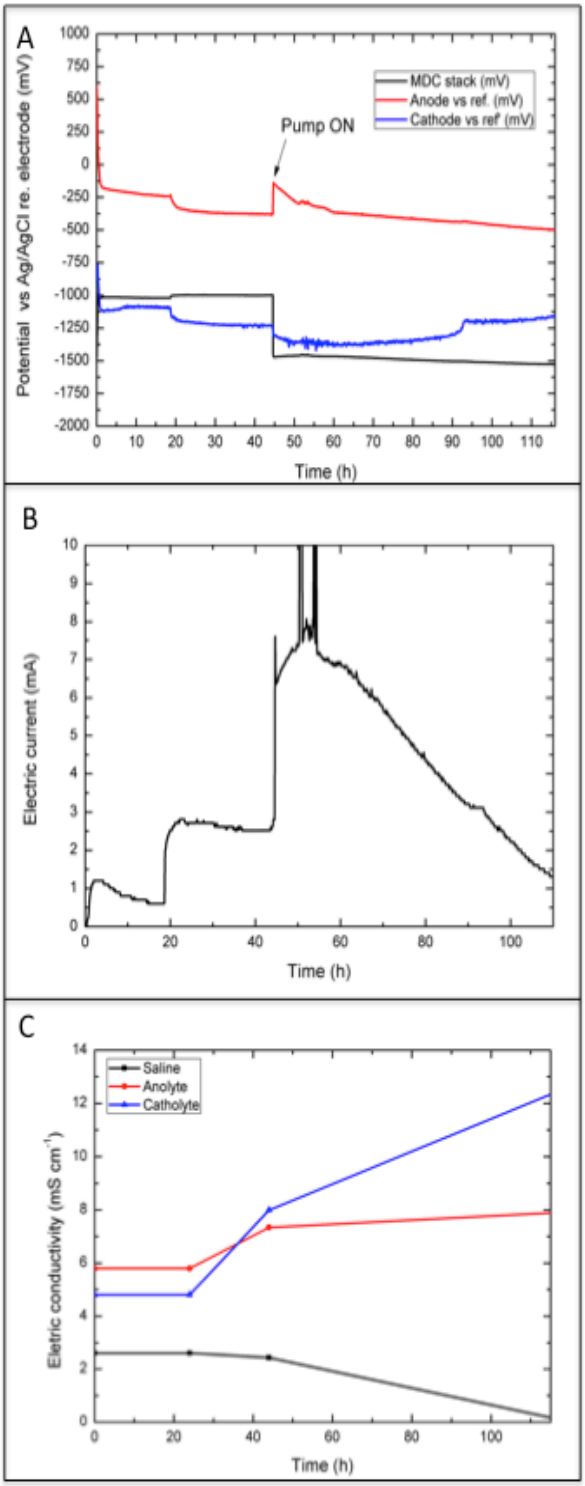
Where E_{cell} is the applied potential (mV), $E_{cathode}$ is the cathode potential (mV), E_{anode} is the anode potential (mV), R_{cell} is the overall resistance of the cell (Ω), and I_{cell} is the electrical current that circulates through the system (mA).

Figure 3. A) Potential for anode, cathode and whole cell; B) Electric current produced during the desalination process; C) Electric conductivity drop in the desalination compartment.

In the case of R_{cell}, its value mainly depends on the electrical resistance of the solutions (anodic, cathodic and saline), and the electrical resistance of the membranes. The electrical current started at a maximum current density of 0.18 mA cm^{-2} and decreased to values around 0.08 mA cm^{-2} after 20 hours of polarization (figure 3B). This behaviour is typical in the conventional electrodialysis systems (Ortiz et al., 2005) operating at a constant applied potential in batch mode because the electric conductivity decreases during the desalination process. Indeed, after 24 hours of operation, a desalination of 68.6 % correlated to a decrease of 75% in the electric current (Figure 3C).

Start-up procedure for the Microbial Desalination Cell

Once the configuration was abiotically tested, the MDC was ready to host a microbial anode. Maintaining the previous cell setup and electrolyte composition, a potential of 1 V was set between electrodes and sterile solutions were recirculated overnight through the two chambers. Then, the anode was inoculated under recirculation-free conditions and the cell was operated in batch mode for 20 hours. During this period (Figure 4) the anode potential dropped from 500 mV to around -300 mV (vs. Ag/AgCl KCl 3.5 M) while the current density increased from the original value to 0.012 mA cm^{-2}. Both phenomena are characteristic of *G. sulfurreducens* electroactive behaviour (Logan, 2008; Malvankar and Lovley, 2012; Schrott et al., 2011) because electric current is obtained from the organic matter oxidation. On the other hand, the cathode potential decreased following the anode potential in order to maintain cell potential (water reduction was the main reaction in the cathode).



After 20 hours of operation, the electric current decreased by 33% (Figure 4B). This bacterial metabolic limitation might be due to a local starvation in electron donor (i.e. acetate) as a consequence of insufficient mixing of the solution. Moreover, local pH decrease can also be a factor that affects bacterial cells performance.

Figure 4. A) Potentials for anode, cathode and whole cell. Black arrow indicates starting recirculation; B) Electric current obtained during the desalination process; C) Electric conductivity in the three chambers (anodic, cathodic and saline chamber)

Even if bicarbonate ions migrate from the desalinization chamber, the lack of flow through the anode chamber could produce internal biofilm acidification as a result of microbial metabolism. These physiological limitations were confirmed once electrolyte recirculation was re-started corresponding to the sudden raise in current generation of 375% (Figure 4B).

After 24 hours of continuous media flow, a stable current output of 2.8 mA was reached (j=0.028 mA cm^{-2}). At the same time, the anode potential decreased indicating insufficient anode polarization. In fact, *G.sulfurreducens* maximal electroactive performance is reported to be achieved at -0.2 V (vs. Ag/AgCl) (Fricke et al., 2008; Richter et al., 2009). When the electrode polarization is below this potential, the biofilm becomes reduced (i.e. charge is accumulated in the cytochromes network) (Bonanni et al., 2013; Robuschi et al., 2013; Schrott et al., 2011). So, in order to increase current production and biofilm discharge, cell voltage was extended from 1 V to 1.5 V, leading to an expected rise in current output of 330% (i.e. from 2,4 to 8 mA) in just 8 h. Moreover, upon repolarization, there was a discharge peak confirming charge accumulation in the cytochrome network due to the capacitor effect of the biofilm (Esteve-Núñez et al., 2008, 2011). Then, current increased from 6 to 8 mA suggesting biofilm growth. Thus, it was evident that a biofilm was colonizing the electrode surface with enough electroactive capacity to drive the desalinisation process whenever sufficient anode polarization was provided. Indeed, electric conductivity of the saline chamber started to decrease significantly till reaching a desalination of 94 % of the initial bicarbonate ion at the end of the desalination cycle (110 h) (Figure 4C).

It is important to note that, once significant desalination was

achieved, the electric current generation showed its highest drop (i.e. after 50 h). This negative impact on the electric conductivity increased the internal resistance and the electric current generation dropped (Figure 4B). It has to be considered that the anode environment was not expected to experience acidification since bicarbonate ions continuously migrated into the anode compartment preserving the pH and conductivity of anode media. Furthermore, the high solution flow rate through the anode chamber (75 mL min^{-1}) ensured adequate media replacement inside the carbon felt anode, minimizing local pH variations. Other factors that could reduce the current generation is the availability of electron donor (i.e. acetate) for *G.sulfurreducens*. However, 70% of the acetate remained during start-up procedure, ensuring adequate availability of substrate for the anodic biofilm. Therefore we conclude that the desalination process caused the decrease in the electric current. An important parameter that determines the efficiency of a MDC device is the volume relationship between the three chambers. So, lower compartments volumes for anolyte and catholyte can be translated into lower energy cost to perform the desalinization process. The desalination performances of several MDC studies, including the current one, are shown in Table 1. Thus, the efficiency of our treatment was still more remarkable considering the low ratio of volumes for anolyte and catholyte compared to the ones reported in other studies. In some cases, the volumes ratios were quadrupled or even 100 times larger than the volumes employed in our system (Cao et al., 2009b; Chen et al., 2011). Studies using similar volume ratio to the ones selected in the current work, reached less than 50% of the desalination obtained in our start-up protocol (Mehanna et al., 2010a, 2010b; Zhang and He, 2015).

V_{an}: V_{cat}: V_{desal}[a]	Start-up protocol	Salt	Removal (%)	COD (%)	Ref.
100:33:1	Transfer of pre-adapted MFC anode	5 $g.l^{-1}$ NaCl	88	-	(Cao et al., 2009b)
36:11:1	Transfer of pre-adapted MFC anode	20 $g.l^{-1}$ NaCl	80	-	(Chen et al., 2011)
4:4:1	1 week anode in-sit stabilization	0.5 $g.l^{-1}$ NaCl	95.8	-	(Zuo et al., 2014)
4:2:1	Inoculation with a mixture sludge	35g.l[1] NaCl	42	74-77	(Zhang and He, 2015)
3:3:1	Transfer of pre-adapted MFC anode	5 $g.l^{-1}$ NaCl	46	25	(Meng et al., 2014)
1.5:1.8:1	Transfer of pre-adapted MFC anode	20 $g.l^{-1}$ NaCl	<84	72-94	(Kim and Logan, 2013b)
1:2:1	Inoculation of a pre-a adapted biofilm	5$g.l^{-1}$ NaCl	43	-	(Mehanna et al., 2010a)
1:2:1	40 days anode in-situ stabilization	20$g.l^{-1}$ NaCl	37[b]	-	Mehanna et al., 2010b)
1:1:1	Bicarbonate desalination	5 $g.l^{-1}$ NaCl	87	53	In this study
	1:1:1	2.5 $g.l^{-1}$ $NaHCO_3$	94[c]	49	In this study

Table 1. [a] V_{an}: anolyte volume, V_{cat}: catholyte volume, V_{desal}: desalinated water volume; [b] With additional voltage of 0.55 V; [c] With additional voltage of 1 V; Removal (%): desalination percentage; COD: percentage of Chemical Oxygen Demand removal. In grey line, conditions and results of the start-up protocol.

Regarding pH change, our catholyte pH experienced a 2-fold increase due to the production of hydroxyl ions from water reduction ($4H_2O$ (l) $+4e^- \rightarrow 2H_2$ (g) $+ 4OH^-$, E^0=-0.42 V). However, previous tests using MDCs suggest that cathode pH was not as critical as anode pH (Zhang et al., 2010) since the latter has an impact on the anodic biofilm growth. During the start-up process there were not any pH variations recorded in the anodic chamber. This supports our previous hypothesis that $NaHCO_3$ was the salt of choice for the start-up protocol (Figure S1). Furthermore, the biological process in the anodic chamber removed 49% of the original COD during the start-up procedure.

Microbial Desalination Process

The start-up procedure was considered to be complete after 100 hours of operation, when an electroactive *G. sulfurreducens* biofilm was developed. To operate in absence of a fixed potential (no power supply), the cathode performance was improved by replacing the previous catholyte with 2 L of NaClO 3% (pH=12). Anolyte was refreshed while the saline solution was changed to a 0.2 L solution of NaCl 5g/l. Finally, the circuit was closed using a shunt resistance (external load) of 2.5 Ohms that allowed monitoring of the electric current through the MDC unit.

During the initial desalination process, electric current production dropped from 7 mA to 3 mA while the anode potential remained stable (Figure 5). The increased internal resistance due to the desalination process was the main reason for this current shift.

Although 20% of the total acetate (enough for microbial growth) was detected in the anolyte after this current drop, anolyte was replaced by new fresh anolyte in order to assure electron donor availability during the whole process. Catholyte was also replaced. Our MDC device was able to desalinate the 65% of the NaCl content in 18 hours. This desalination rate was higher than the one reported in a similar volume ratio after 18 days of pre-acclimatization (Meng et al., 2014) (Table 1).

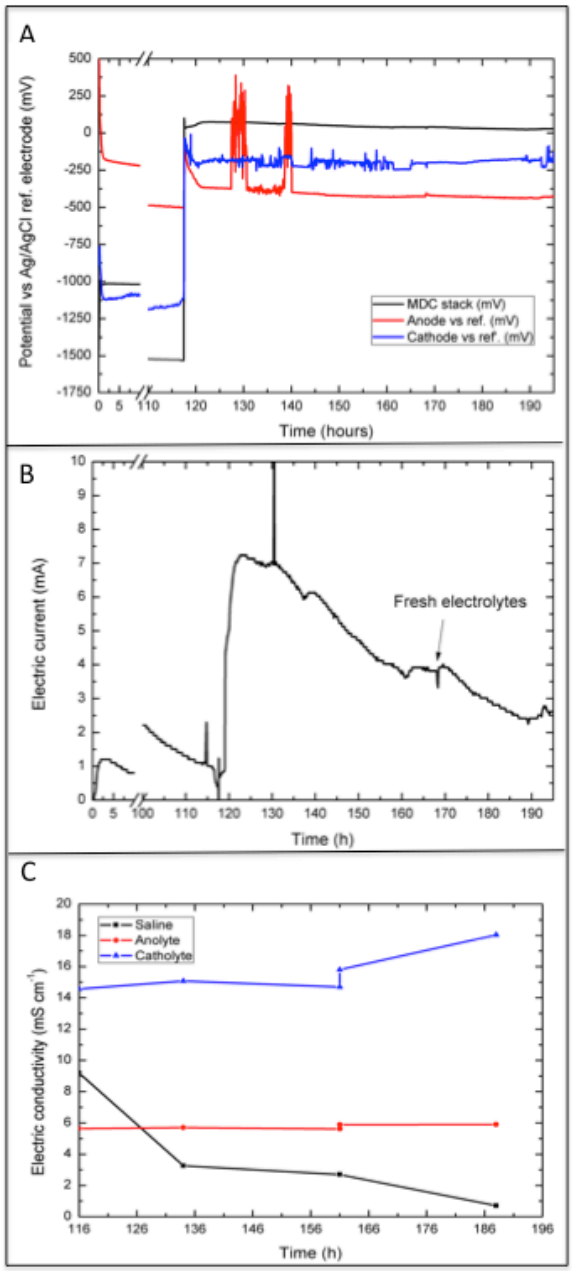


Figure 5. A) Potential for anode, cathode and whole cell; B) Electric current produced during the desalination process. Black arrow indicates time of replacing anolyte and catholyte; C) Electric conductivity of the anodic, cathodic and saline streams.

Operating the MDC (no power supply) for 65 hours resulted in ionic conductivity 10 times lower than the original saline solution (1mS cm^{-1}, potable water limit) (Figure 5C). At the same time, both the anolyte and the catholyte conductivity remained stable, mainly due to the high ratio anolyte/saline volume and/or catholyte/saline volume. This ratio was key to avoid the generation of brine, one of the main environmental problems associated to reverse osmosis.

Another key aspect for a proper performance of the microbial biofilm on the anode is the pH. In that sense, both anolyte and catholyte pH slightly increase in less than one unit allowing the maintenance of the anodic biofilm (Figure S2). Actually, COD removal was increased from 49 % under power supply conditions to 53% under the MDC operation mode.

Including the start-up procedure of the system, a complete desalination cycle with a successful desalination rate of 87% was performed in a total operation time of 196 h. Our system was able to desalinate in a time period similar to the times reported elsewhere for adapting the microorganisms before starting the MDC operation (Zuo et al., 2014), in some cases, the adaptation period was even duplicated (Meng et al., 2014). Although the pre-adaptation time of the anode could be significantly reduced, the start-up protocols used by other studies have two main drawbacks: the need of pre-running as a MFC and disassembly for incorporating the anode into the MDC. In this work, the start-up protocol presented is optimized for time and has a simplified operation, making it a more feasible strategy for future scaling-up. Finally, the start-up protocol presented could be used to systematic study different MDC configurations for performance and optimize MDC experimental set-ups.

CONCLUSIONS

Microbial Desalination Cells constitute an innovative technology where microbial fuel cell and electrodialysis merge into one device for obtaining fresh water from saline water with no associated energy cost. However, a number of factors should be further explored to optimize this methodology and to make it profitable. Although some parameters such as electrode materials, membranes and design are the conventional issues under research, here is presented a new strategy for the start-up procedure. The presented protocol allowed obtaining an optimal and robust biofilm able to operate in the MDC without power supply while achieving 87 % desalination in just 3 days. In this way, the viability of the start-up procedure as well as the modular nature of our MDC prototype was demonstrated. The start-up protocol presented here, could allow our MDC prototype to be a real solution for scaling-up an autonomous process as a pre-treatment method (Brastad and He, 2013) for other well established desalination technologies such as reverse osmosis (RO) (Mehanna et al., 2010a) or reverse electrodialysis (Wallack et al., 2015).

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SUPPLEMENTARY INFORMATION

Figure S1. pH from the anolyte, catholyte and saline streams during the start-up procedure of the MDC prototype.

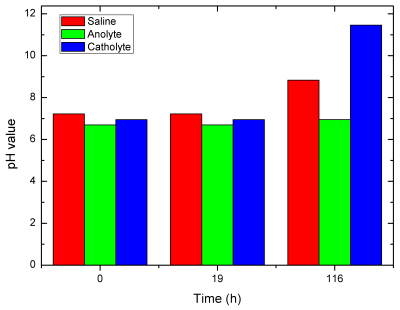
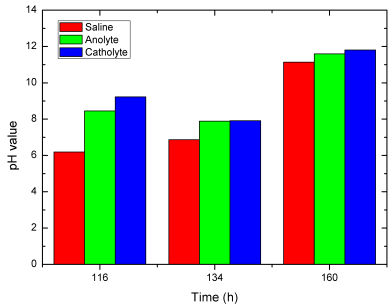
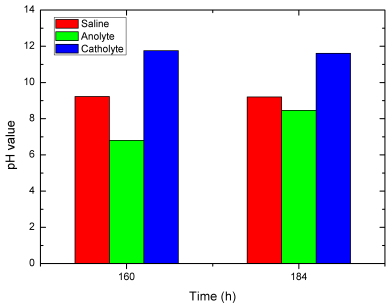


Figure S2. pH from the anolyte, catholyte and saline streams during desalination process (left). Values of the refreshed anolyte and catholyte (right).





Discussion and General Conclusions/ Discusión y Conclusiones Generales

DISCUSSION AND GENERAL CONCLUSIONS

The discipline of microbial electrochemistry has greatly evolved since the discovery of the electroactive microorganisms (Bond et al., 2002) and it has been mainly developed associated to promising applications in the environmental area. The platform of technologies behind that discipline is so-called Microbial Electrochemical Technologies (METs). It comprises a set of alternatives of microbial biotechnology applications with a strong energetic and environmental component (Schröder et al., 2015).

Like any technological development, METs aims to become a practical reality. To do this, it must undergo intense study and analysis, first for its optimization and later, for it scaling-up. With this aim, this thesis has focused on the study of the essential core of the METs, the electroactive bacteria.

Since their discovery, the paradigm of study of the electroactive microorganisms it has been based on the biofilm structure (Bonanni et al., 2013) because, under this circumstances, it is allowed the artificial condition of respiring an electron acceptor, which nature is being an insoluble material with a certain area. On the contrary, in their natural habitat, *Geobacter sulfurreducens* (object of study in this thesis), is found in planktonic state (Childers et al., 2002; Holmes et al., 2002). It is interesting to note that in planktonic state, *G.sulfurreducens* has a weak electroactive phenotype. However, electroactivity can be enhanced when cells are structured in several layers within a biofilm. For this same reason, the goal of this thesis has been the study of *G.sulfurreducens* physiology in planktonic state

and in absence of electrode with the objective of promoting a more electroactive phenotype.

Many complex strategies (e.g. genetic manipulation) for obtaining a highly electroactive phenotype were initially proposed by other authors (Leang et al., 2013). However, we have employed a broader approach by cultivation of planktonic *G.sulfurreducens* in a continuous mode culture with a kind of bioreactor known as chemostat (Esteve-Nunez et al., 2005). Growing *Geobacter* under this condition and under electron acceptor limitation (electron donor excess), it has been achieved planktonic microorganisms with a higher electroactivity. We have denominate this microbial phenotype as *plug and play* cells due to their ability to produce electric current at the first contact with the electrode.

It has been reported that these cells overexpress C-type cytochromes (Esteve-Núñez et al., 2011) allowing a greater connection with the electrode. This larger production of external cytochromes allowed for discovering the *capacitor* phenomenon (Esteve-Núñez et al., 2008) that, through the vast network of multi-heme cytochromes, has the ability of accumulate the electrons produced in the oxidative metabolism. This process made the cytochromes network to be known as the metaphoric term *iron lungs* (Lovley, 2008). In this way, under the lack of any acceptor, the *lungs* would be of filled electrons conferring to the cells a charged (electric) state. This storage ability can be quantified with the measurement of discharge in either a chemical acceptor like a quinone (Esteve-Núñez et al., 2008) or a polarized electrode (Bonanni et al., 2012).

Even though *capacitor* effect demonstrates an intrinsic ability of the cytochromes network to accumulate charge, our work hypothesis for the obtaining of electroavice planktonic cells, is based on the encourage of the opposite reaction, the electric discharge. Our hypothesis has been supported by the results obtained in Chapter 2, in which current is immediately produced upon contact to the polarized electrode (Borjas et al., 2015). The efficiency of *plug and play* cells, that was successfully tested in a pre-pilot scale prototype capable of coupling electrical energy production with acetogenic wastewater treatment, allowed us to significantly reduced start-up times as well as COD removal. As in the *capacitor* effect the whole multiheme cytochromes is the responsible of the electrons storage, in the *plug and play* cells the discharge reaction requires a specific cytochrome: OmcB. The role of this cytochrome in the respiration of extracellular acceptors like the Fe (III), it has already been demonstrated (Leang et al., 2003). Nevertheless, in this thesis it is reported for the first time the OmcB implication in the respiration of electrodes by planktonic cells.

Other application area of METs, through the MDC (Microbial Desalination Cells), is the desalination one. The MDC can suppose an alternative in the desalination area as they combine water treatment and desalination in just one step. However, to be a real alternative to the expensive membrane technologies, several factors have to be further analysed to optimize their performance and therefore, their profitability. One of these parameters is the adaptation of electroactive microorganisms to a saline environment, which can be found in the anodic chamber of these devices (Kim and Logan, 2013; Libin Zhang, 2011). For this reason, *G.sulfurreducens* was cultivated

under saline stress in order to validate it as electroactive microorganism for MDC. Surprisingly, the saline stress triggered an osmotic response that led to an increased current output. The osmoregulation strategy developed by the microorganism was studied in depth by the comparison of its proteome with other microorganisms, which were under similar conditions (Oren, 2008; Qurashi and Sabri, 2012; Teng et al., 2015). Among the results of the study, it should be pointed out that the exposure to salt medium mainly involved an exacerbated synthesis of extracellular polymeric substances (EPS). The isolation of this EPS matrix enriched in redox proteins, with the proteome analyses, allowed us to detect a higher abundance of redox proteins like the cytochrome OmcS, which redox activity on electrodes has been demonstrated (Leang et al., 2010).

Though other authors (Strycharz-Glaven et al., 2014) have previously proposed the role of EPS within the biofilm, our approach has been the study of it in a new scenario, the planktonic cells one. The isolation of the EPS matrix and its further incubation with poorly-electroactive microorganism, allowed us to obtain a more electroactive bacteria and, therefore to confirm the key role of EPS in the electrocatalytic activity. These results demonstrated not only the viability of *G.sulfurreducens* to be used in MDC systems, but also a new physiologic approach based on saline stress for obtaining highly electroactive microorganism without neither the presence of an electrode nor the genetic manipulation of the strain (Chapter 2). Furthermore, the possibility of isolating and transferring the EPS matrix let us to propose it as a novel conductive biological material that can yield improved bioelectronics material with energy applications.

Another fundamental factor in the MDCs optimization is the start-up time of the systems. This process commonly involves the external adaptation of the electroactive microorganism, which is subsequently included in the MDC system (Mehanna et al., 2010; Meng et al., 2014). This strategy not only involves an extra amount of time but also entails a technical obstacle to a real scaling-up of the technology. In this thesis, we explored other possibilities posing a *in situ* start-up procedure that allowed us to desalinate from the first phase of the system operation (Chapter 3). In addition, the volume ratio between electrolytes and saline stream allowed in our system avoid the generation of brine, which in other desalination technologies supposes a negative environmental impact when it comes to discharge it. This environmental advantage as well as the modular design of the pre-pilot prototype used, made MDC a real proposal for scaling-up to operate both individually and in combination, as a pre-treatment for other desalination technologies. In fact, this proposal has been represented in MIDES project of the H2020 programme, which is going to give continuation to the methods proposed in this thesis

Therefore, it can be concluded that:

1. It has been demonstrated the electroactivity efficiency of *plug and play G.sulfurreducens* cells reducing start-up times in a pre-pilot system coupled to the acetogenic wastewater treatment. This electroactive efficiency is in relation to a greater abundance of the outer membrane C-type cytochrome, OmcB.

2. We have developed a simple approach for obtaining highly

electroactive *G.sulfurreducens* by growing them under salt stress.

3. Both *plug and play* cells and salt-grown cells of *G.sulfurreducens* demonstrate not only the key role of physiology in the electroactivity optimization, but also the possibility of obtaining highly-electroactive cells without the growth of a biofilm. Salt-grown cells facilitate the use of microbial electrochemistry in other disciplines where there are not an enough microbiological and technological components for biofilm growth.

4. It has been shown that the resulting osmotic response of salt-grown cells is closely related to such enhanced electroactivity due to changes in the EPS profile. A higher abundance of the extracellular cytochrome OmcS, which is involved in the electron transfer process, seems to be in relation with this increased electroactive response. Further analyses in the composition of the EPS matrix are required.

5. The merge of conventional electrodialysis with others from MDC technology has lead to an optimal start-up procedure that contribute to the scaling-up of the system.

DISCUSIÓN Y CONCLUSIONES GENERALES

La electroquímica microbiana ha experimentado una gran evolución desde el descubrimiento de los microorganismos electroactivos (Bond et al., 2002), impulsada principalmente por el desarrollo de aplicaciones prometedoras dentro del área medioambiental. La plataforma de tecnologías que explotan dicha disciplina se conoce como MET (del inglés Microbial Electrochemical Technologies) y comprenden un conjunto de aplicaciones bioenergéticas en el campo de la biotecnología microbiana (Schröder et al., 2015).

Como todo desarrollo tecnológico, el fin último es el de llegar a ser una realidad práctica, y para ello ha de someterse a un intenso estudio y análisis para lograr su optimización y, posteriormente, para consumar su escalado. Con el objeto de optimizar la tecnología, esta tesis se ha centrado en el estudio de los actores principales de las METs, los microorganismos electroactivos.

Desde su descubrimiento, el paradigma de estudio de los microorganismos electroactivos se ha basado en la estructura de biofilm (Bonanni et al., 2013) puesto que, a través de ella, se permite la condición artificial de poder respirar un aceptor cuya naturaleza es la de ser un material insoluble con una determinada área superficial. Al contrario, en su hábitat natural, el microorganismo electroactivo modelo *Geobacter sulfurreducens* (objeto de estudio en esta tesis) se encuentra en estado planctónico (Childers et al., 2002; Holmes et al., 2002). Es interesante destacar que, en estado planctónico *G.sulfurreducens* presenta una respuesta electroactiva baja, que se ve potenciada cuando crece estructurado en varias capas celulares dentro de un biofilm. Por esta misma razón, el objetivo principal de

esta tesis ha sido el estudio de la fisiología de dicho microorganismo en estado planctónico y en ausencia de electrodo, con la finalidad de explorar la posible inducción de un fenotipo más electroactivo.

Hasta ahora, muchas han sido las estrategias complejas (por ejemplo, la ingeniería genética) desarrolladas por otros grupos de investigación (Leang et al., 2013). Sin embargo, nosotros hemos diseñado un método más sencillo recurriendo al cultivo de *Geobacter* en modo continuo mediante un tipo de biorreactor denominado quimiostato (Esteve-Nunez et al., 2005). En estas condiciones y bajo una limitación de aceptor de electrones (exceso de donador de electrones), se han logrado bacterias planctónicas que exhiben mayor electroactividad. A este fenotipo bacteriano lo hemos denominado como bacterias *plug and play,* por su capacidad para generar corriente eléctrica al primer contacto con el electrodo.

Se ha descrito que estas células sobre-expresan citocromos tipo C (Esteve-Núñez et al., 2011) permitiendo una mayor conexión electroquímica con el electrodo. Esta mayor producción de citocromos externos permitió descubrir el fenómeno capacitor (Esteve-Núñez et al., 2008), a través del cual la extensa red de citocromos multihemo tendría la capacidad para almacenar los electrones generados en el metabolismo oxidativo. Este nuevo proceso llevó a que la red de citocromos fuera bautizada con el metafórico término de *iron lungs* (pulmones de hierro) (Lovley, 2008). De esta forma, en ausencia de aceptores externos los *pulmones* se irían llenando de electrones confiriendo a la célula un estado de carga (eléctrica). Dicha capacidad de almacenamiento puede ser cuantificada midiendo la magnitud de la descarga ante un

aceptor químico como una quinona (Esteve-Núñez et al., 2008) o bien un electrodo polarizado (Bonanni et al., 2012).

Si bien el efecto capacitor demuestra una capacidad intrínseca de la red de citocromos para almacenar carga, nuestra hipótesis de trabajo para obtener células planctónicas electroactivas se basa en fomentar la reacción contraria, la de descarga. Nuestra hipótesis se ha visto respaldada por los resultados del capítulo 2, en los que la corriente eléctrica es generada por las células planctónicas al contactar con el electrodo (Borjas et al., 2015). La eficiencia de las bacterias *plug and pl*ay, ha sido cuantificada en un prototipo a escala pre-piloto, capaz de acoplar la producción de energía eléctrica con el tratamiento de aguas residuales acetogénicas permitiendo reducir tanto los tiempos de operación como la eliminación de DQO. Si en el efecto capacitor es el conjunto de los citocromos mutihemo el responsable del almacenamiento de los electrones, en las células *plug and play* la reacción de descarga requiere la presencia de un citocromo específico: el OmcB. El papel de este citocromo en la respiración de otros aceptores extracelulares como el Fe(III) ya había sido demostrado (Leang et al., 2003) pero en esta tesis se muestra por primera vez su implicación en la respiración de electrodos por parte de células en estado planctónico.

Otro campo de aplicación de las MET, a través de las MDC (del inglés Microbial Desalination Cell), es el de la desalinización (Cao et al., 2009; Chen et al., 2011). Las MDC podrían suponer una alternativa en el área de la desalinización puesto que combinan de forma simultánea, el tratamiento de aguas y la desalinización. Sin embargo, para que sea una alternativa real a las costosas tecnologías de membranas actuales, varios factores han de ser aún analizados con

el objeto de optimizar su operación y su rentabilidad. Entre estos parámetros se encuentra la vulnerabilidad de los microorganismos electroactivos a las condiciones salinas que se pueden dar en las cámaras anódicas de estos dispositivos (Kim and Logan, 2013; Libin Zhang, 2011). Por este motivo, *G.sulfurreducens* se sometió a estrés salino con el objeto de validarlo como microorganismo electroactivo para ser empleado en MDC. Sorprendentemente, el estrés salino disparó una respuesta osmótica que conllevó el aumento en la electroactividad y, por tanto, en la producción de corriente. La estrategia osmoadaptativa mostrada por el microorganismo fue estudiada en profundidad a partir del análisis de su proteoma, comparándola con la encontrada en otros microorganismos (Oren, 2008; Qurashi and Sabri, 2012; Teng et al., 2015). Como resultado del estudio, cabe destacar que el crecimiento en medio salino implicó una síntesis exacerbada de sustancias extracelulares poliméricas (EPS). El aislamiento de esta matriz rica en EPS, junto al análisis de su proteoma, permitió detectar una mayor abundancia de proteínas redox como el citocromo OmcS, uno de los citocromos con probada actividad con electrodos (Leang et al., 2010).

Si bien otros autores (Strycharz-Glaven et al., 2014) han sugerido previamente el papel del EPS dentro de los biofilm electroactivos, nuestro enfoque ha sido el de estudiar su papel en un nuevo escenario, el de las células planctónicas. El aislamiento de la matriz de EPS y su posterior incubación con células menos electroactivas, nos permitió obtener una fisiología bacteriana de mayor electroactividad, sugiriendo así un papel clave de los EPS en la actividad electrocatalítica. Estos resultados demostraron, no solo la viabilidad de *G.sulfurreducens* para ser empleado en sistemas de MDC, sino también una nueva estrategia fisiológica basada en estrés salino

para la obtención de bacterias más electroactivas cultivadas incluso en ausencia de electrodos (Capítulo 2). Además, la posibilidad de aislar y transferir la matriz de EPS, nos ha permitido proponerla como un nuevo material biológico conductor que podría optimizar materiales biológicos con aplicaciones bioelectroquímicas.

Otro factor clave en la optimización de las MDC es el tiempo de arranque de los sistemas. Este proceso, conlleva la adaptación externa del microorganismo que posteriormente es incluido en el sistema (Mehanna et al., 2010; Meng et al., 2014). Esta estrategia no solo implica un cantidad extra de tiempo sino que supone un obstáculo técnico para un escalado real de la tecnología. En esta tesis exploramos otras posibilidades planteando un procedimiento de arranque *in situ* que permitió desalinizar desde la primera fase de la operación del sistema (Capítulo 3). Además, la proporción de volúmenes entre los electrolitos y la solución salina permitieron evitar la generación de salmueras, con la consiguiente reducción de impacto medioambiental. Esta mejora, junto al diseño modular de nuestro sistema pre-piloto, hacen de las MDC una propuesta real para ser utilizadas de forma individual o combinada, como un pre-tratamiento para otras tecnologías de desalinización. De hecho, esta propuesta se ha visto reflejada en el proyecto MIDES (Microbial Desalination) del programa H2020 asegurando la continuación de los métodos de esta tesis.

Por tanto, se puede concluir que:

1.- Se ha demostrado la eficiencia electroactiva de las bacterias *plug and play* de *G.sulfurreducens.* Se ha disminuido los tiempos de operación un sistema pre-piloto acoplado a la depuración de aguas acetogénicas usando dichas células como inóculo. La eficiencia electroactiva del microorganismo requiere la presencia del citocromo C de membrana externa, OmcB.

2.- Se ha desarrollado un protocolo de cultivo sencillo para la obtención de células de *G.sulfurreducens* de alta electroactividad, mediante su cultivo anaeorbio bajo estrés salino.

3.- Tanto la obtención de bacterias de *G.sulfurreducen,s* con una fisiología *plug and* play, como las cultivadas bajo estrés salino, han demostrado la influencia del medio de cultivo sobre la electroactividad microbiana en sistemas sin electrodos. Asimismo se ha demostrado que es factible obtener bacterias planctónicas de alta electroactividad sin necesidad de inducir el crecimiento de un biofilm. Además, las células cultivadas en medio salino facilitan el uso de la electroquímica microbiana en otras disciplinas.

4.- Se ha demostrado que la respuesta osmótica de las células cultivadas en sal, está íntimamente relacionada con el aumento de electroactividad mediante el cambio en el perfil de los EPS sintetizados. Una mayor abundancia del citocromo de membrana externa OmcS, implicado en la transferencia de electrones, parece estar vinculado al aumento de la electroactividad.

5.- La fusión de metodologías de la electrodiálisis convencional con otras más propias de las MDC ha dado lugar a un protocolo de arranque que favorece el escalado del sistema.

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