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CONSTRUCTED WETLAND MICROBIAL FUEL CELLS
Electricity generation, treatment efficiency improvement, COD bioindication and clogging assessment

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THESIS DISSERTATION OF THE PHD TITLE

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Summary

Horizontal Subsurface Flow Constructed Wetlands (HSSF CWs) are natural wastewater treatment systems showing a marked redox gradient between the surface of the system and the bottom zone of the treatment bed. Therefore, they constitute a suitable environment for Microbial Fuel Cells (MFCs) implementation. MFCs are bioelectrochemical systems in which the electrons resulting from the oxidation of the organic matter are transferred, by means of exoelectrogenic bacteria, to an external circuit thus generating an electric current. The implementation of MFC into HSSF CWs (CW-MFCs) allows the removal of organic matter and the production of electricity simultaneously. Besides electricity generation, MFCs implemented in HSSF CWs could encompass other beneficial aspects of special relevance within the constructed wetlands domain such as the semi-continuous monitoring of the organic matter entering the system, the improvement of CWs' treatment efficiency and the assessment of the clogging state of the treatment bed. However, CW-MFCs is a novel research field that lacks from specific knowledge on both HSSF CWs and MFC design and operational aspects to optimize the technology. Therefore, the objective of this thesis was to

determine, quantify and maximize the benefits resulting from the synergy between HSSF CW and MFCs.

To address the objectives of this study two different experimental designs were considered. The optimization of both HSSF CW operation conditions and MFCs architecture was assessed by implementing CW-MFCs within a pilot-scale treatment plant. The pilot plant consisted of 8 HSSF CWs operated at different conditions: hydraulic regime (continuous and batch), presence of plants (planted or unplanted), primary treatment (hydrolytic sludge blanket reactor - HUSB reactor - vs conventional settler) or different organic loadings. MFCs implemented in the pilot plant had graphite rod electrodes and were placed within the gravel bed. The experimental set-up allowed us the measurement of the redox gradients established within the gravel bed and the voltage generated by MFCs under these conditions. The experiments regarding the quantification of the treatment improvement and the monitoring capacity (chemical oxygen demand - COD - and clogging) of CW-MFCs were carried out by means lab-scale air-cathode CW-MFCs. Also lab-scale CW-MFCs were used to evaluate graphite and gravel as different anodic materials.

Results showed that continuous flow regime and planted wetlands generate higher redox gradients through the gravel bed than unplanted wetlands operated under discontinuous flow regime. CW-MFCs performed to a better extent under the presence of a HUSB reactor as primary treatment when compared to conventional settling. More precisely, the presence of HUSB reactor stimulated the presence of exoelectrogenic bacteria in anodic biofilms. Optimal cathode to anode surface ratio was that of 4:1. In order to maximize CW-MFCs performance, the cathode shall be placed semi-submerged within the water and kept at a distance of ca. 10 cm from the anode. Overall, even under these optimal wetlands and MFC operational and design conditions the energy produced by CW-MFCs would only cover between the 3 and the 14 % of the total energy requirements of a CW treatment plant. Therefore, energy surplus provided by CW-MFCs, yet interesting, is not its most advantageous feature.

In terms of CW-MFCs environmental applications, MFCs showed the capacity to improve CWs treatment efficiency. Organic matter effluent

concentration for connected CW-MFCs was significantly lower both in terms of total and soluble COD than unconnected CW-MFCs. Also ammonia and phosphates removal was stimulated in connected systems. More into detail, MFCs operated at closed circuit showed ca. 21%, 18%, 15%, 31% and 25% lower effluent concentration than unconnected MFCs to the BOD_5, COD, TOC, PO_4^{-3} and NH_4^+-N, respectively. Furthermore, CW-MFCs showed potential for COD assessment. Accordingly, a positive linear relationship between the inlet COD and the electrical current generated could be stablished. Although results indicate that linear relationships can be established between both parameters, several factors can affect the precision, repeatability and operational stability of the sensor. Therefore, other alternatives such as its utilization as qualitative assessment tools should be considered for biosensor CW-MFCs. Finally, CW-MFCs also showed potential as a tool for indirect, continuous clogging assessment.

In terms of the environmental impacts associated to the implementation of MFCs, a CW system coupled with a graphite-based anode MFC appeared as the most environmentally friendly solution which could reduce CW both surface requirements and system footprint (by around 20%). Furthermore, CW systems coupled with high performance MFCs would be competitive with conventional CWs in terms of costs.

In conclusion, although still in its infancy, CW-MFCs represent a novel technology able produce energy while wastewater is treated. Although it might not be a very attractive technology if the electrical gain is considered exclusively, CW-MFCs is a very promising technology when it comes down to environmental applications such as the improvement of HSSF CWs removal efficiency, or the utilization of CW-MFCs as both organic matter and clogging assessment tool.

Resum

Els aiguamolls construïts horitzontals de flux subsuperficial (AC HFSS) son sistemes naturals de tractament d'aigua residual. En aquests sistemes es genera un gradient redox entre la superfície de l'aiguamoll i les capes profundes del mateix. Així doncs, constitueixen un entorn favorable per a la implementació de Piles de Combustible Microbianes (PCM). Les PCM son sistemes bioelectroquímics en els que els electrons que resulten de la oxidació de la matèria orgànica són transferits per bactèries exoelectrogèniques a un circuit extern de forma que es genera un corrent elèctric. La implementació de les PCM als AC HFSS (PCM-AC), permet doncs la eliminació de la matèria orgànica i la generació d'electricitat de forma simultània. A més a més de la generació d'electricitat, les PCM implementades en AC, poden generar altres beneficis que són d'especial importància en el context dels AC. El monitoreig semi-continu de la qualitat de l'aigua que entra i surt dels sistemes, la millora de la eficiència de tractament dels AC o el seguiment de l'estat de colmatació del llit de grava dels AC són alguns dels potencials beneficis resultants. Tot i això, les PCM implementades en AC, constitueixen un camp de recerca molt recent en el que encara falten coneixements específics tant sobre el disseny dels AC com sobre la operació de les PCM per optimitzar la sinèrgia entre ambdues tecnologies. Així doncs, l'objectiu de la tesi que es presenta és el de determinar, quantificar i maximitzar els beneficis que resulten de la sinergia entre els AC HFSS i les PCM.

Per assolir els objectius d'aquest estudi s'han utilitzat dos dissenys experimentals diferents. La optimització de les condicions d'operació dels AC i de l'arquitectura de les PCM s'ha estudiat mitjançant la implementació de les PCM en una planta d'AC a escala pilot. La planta pilot consistia en 8 AC HFSS operats amb diferents condicions de: règim hidràulic (continu i discontinu), presència de plantes (plantats i no-plantats), tractament primari (reactor hidrolític de flux ascendent – reactor HUSB- i decantador convencional) i càrregues orgàniques diferents. Les múltiples condicions d'operació aplicades han permès la mesura dels gradients redox establerts en el llit de grava i del voltatge generat per les PCM implementades. Les PCM implementades a la planta pilot estaven constituïdes per elèctrodes fets amb cilindres de grafit i estaven ubicades a l'interior del llit de grava dels AC. En segon lloc, els experiments que tenien l'objectiu de quantificar la millora de la capacitat de tractament dels AC com a conseqüència de la implementació de les PCM i de determinar la capacitat de monitoreig de les mateixes (en termes de demanda bioquímica d'oxigen – DQO- i de colmatació del llit de grava) es van dur a terme mitjançant PCM-AC a escala de laboratori. Aquest segon disseny experimental també va ser utilitzat per avaluar la diferència entre el grafit i la grava en tant que materials anòdics.

Els resultats obtinguts demostren que el flux hidràulic continu i els aiguamolls plantats generen gradients redox superiors en el llit de grava quan es comparen amb els aiguamolls no-plantats operats en flux discontinu. Les PCM-AC van generar corrents elèctrics superiors quan el tractament primari aplicat fou el reactor HUSB en comparació al decantador convencional. De fet, l'aplicació d'un reactor HUSB en tant que tractament primari de l'aigua residual va estimular la presència de bactèries exoelectrogèniques als biofilms anòdics. La relació òptima de superfície càtode : ànode obtinguda fou de 4:1. A més a més, per tal de maximitzar el potencial de les PCM-AC, s'ha demostrat que cal situar el càtode en posició semi-submergida i a una distància de 10 cm de l'ànode. Tot i això, quan s'apliquen les condicions d'operació dels AC i l'arquitectura de les PCM òptima, l'energia produïda per les PCM-AC pot cobrir només entre el 3 i el 14% d'una planta de tractament d'aigua residual constituïda per aiguamolls construïts. Així doncs, tot i que l'energia generada per les PCM-AC constitueix un resultat positiu, no esdevé el benefici principal.

En termes de les seves aplicacions ambientals, les PCM-AC han demostrat poder millorar la capacitat de tractament dels AC. La concentració de matèria orgànica a l'efluent de les PCM-AC connectades fou significativament inferior que a l'efluent dels sistemes no connectats tant en termes de DQO total com soluble. Aquest resultat suggereix que els biofilms electroactius van estimular la degradació de la matèria orgànica en els sistemes connectats. A més a més, les PCM-AC han mostrat potencial per a la determinació de la DQO. Així concs, ha estat possible establir una relació lineal positiva entre la DQO d'entrada a les PCM i l'electricitat generada per les mateixes. Tot i això, encara que els resultats d'ambdós paràmetres segueixen una relació lineal, hi ha molts factors que poden afectar la precisió, la repetibilitat i la estabilitat operativa del sensor. És per això que cal considerar altres alternatives com la seva utilització com a eines de resposta qualitativa. Per últim, les PCM-AC han demostrat tenir també potencial per a la determinació en continu del grau de colmatació dins el llit de grava dels AC.

De l'estudi dels impactes ambientals associats a la implementació de les PCM en els AC se'n conclou que els sistemes amb l'ànode de grafit constitueixen la millor alternativa en termes d'impactes ambientals per reduir tan la superfície dels aiguamolls com la petjada ecològica (sobre un 20% de reducció). En termes de costos, cal destacar que tot i el cost elevat dels materials utilitzats (grafit i acer inoxidable principalment), la sinergia entre els AC i les PCM d'alta eficiència constituirien una alternativa competitiva.

Finalment, tot i trobar-se en un estadi d'estudi prematur, les PCM implementades en AC representen una tecnologia innovadora capaç de produir energia alhora que es tracta l'aigua residual. Encara que, si es considera de forma exclusiva el guany energètic, la sinèrgia entre ambdues tecnologies pot no semblar molt atractiva, les PCM-AC constitueixen una tecnologia molt prometedora pel que fa a les seves aplicacions ambientals tals com la millora de la eficiència de tractament dels AC o la seva utilització per a la determinació del contingut de matèria orgànica en l'aigua residual i el grau de colmatació dels sistemes.

Introduction

Constructed wetlands (CW) are natural wastewater treatment systems that constitute an excellent alternative to conventional technologies for the sanitation of small communities. In constructed wetlands wastewater is treated by means of physical, chemical and biological processes taking place inside the treatment bed. The presence of organic matter (OM) and the naturally generated redox gradient between the upper layer (in contact with the atmosphere) and the deeper layers (completely anaerobic) constitute a suitable environment to implement Microbial Fuel Cells (MFCs). MFCs is a technology that generates electricity from the microbial degradation of organic and inorganic substrates. In a MFC, OM is oxidised by microorganisms in an anaerobic compartment and the electrons produced during the oxidation are transferred to the electrode (anode). By means of an external and conductive circuit, electrons flow to reduce an electron acceptor,

such as oxygen, at the cathode. Therefore, electricity is generated at the time that the organic matter is degraded.

Although it is a new research field, MFCs implemented in CW has recently received notable attention. MFC implemented in CW (CW-MFCs) will not only produce an energy surplus while wastewater is treated but will also contribute to improve and monitor the overall treatment process. CW-MFCs energy production would be of special interest within the constructed wetlands scenario, since one of the major advantages of this technology is the low energy input necessary for the wastewater treatment. Accordingly, the implementation of MFCs in constructed wetlands can result in the generation of electricity to cover a part of the treatment plant energy requirements. Besides the benefits that an extra energy source for the treatment system could provide, the implementation of MFCs may exert other beneficial effects on CWs directly linked to the minimization of CWs major drawbacks. To this regard, CWs, and especially horizontal subsurface flow CW (HSSF CWs), have relatively larger surface requirements when compared with conventional treatment technologies and suffer from a progressive reduction of hydraulic conductivity (clogging). During the last years, research in HSSF CWs has focused on the improvement of treatment performances, surface requirements reduction and clogging delay. Among the strategies to increase CWs performances and reduce surface, forced aeration has been of special importance. However, active aeration results in large energy consumption. Main strategies for clogging remediation include the application of chemicals and gravel replacement. However, such strategies increase the overall plant operation cost.

MFCs implemented in constructed wetlands may have a significant contribution on improving treatment capacity by fostering more efficient degradation pathways. Also CW-MFCs would be a suitable bioelectrochemical tool for the assessment of treatment performance without any additional cost involved in the process. Accordingly, both the organic matter pollution in wastewater and the clogging state of wetlands' gravel bed shall be assessed by means of MFCs. All the beneficial aspects encompassing the application of microbial fuel cells to wetlands are of capital interest for the optimization of such technology. CW-MFCs is a novel research field that lacks from specific knowledge on both HSSF CWs and MFC design and

operational aspects to optimize the technology. Currently one of the challenges is to determine which operational and design criteria in constructed wetlands optimize the performance of microbial fuel cells. Accordingly, there are many operational conditions in wetlands that have a certain influence on redox conditions within the treatment bed and, thus, can affect MFCs' performance, such as the presence of macrophytes, the feeding strategy or the primary treatment applied, among others. Also MFCs' architecture itself shall be adapted for its implementation in constructed wetlands either to be optimized for energy production, treatment efficiency improvement, wastewater organic pollution bioindication or clogging assessment. Due to its novelty the environmental and economic impacts of implementing MFCs in CWs are also still unaddressed. The study of the impacts associated is of great importance especially when MFCs are implemented in such a natural and low-cost technology like HSSF CWs. Therefore, the main objective of this thesis was to determine, quantify and maximize the benefits resulting from the synergy between horizontal subsurface constructed wetlands and microbial fuel cells.

CHAPTER 2

Objectives

The main **objective** of this thesis was to determine, quantify and maximize the **benefits** resulting from the **synergy** between horizontal subsurface **constructed wetlands** and **microbial fuel cells**.

OB (1)   To determine the influence of **CWs operational conditions** on energy production in CW-MFCs.

OB (2)   To determine the influence of **MFC architechture** on energy production in CW-MFCs.

OB (3)   To determine and quantify the effect of the implementation of MFCs on CWs **treatment efficiency**.

OB (4)   To determine the applicability of CW-MFCs as a **COD bioindication tool** in wastewater.

OB (5)   To determine the applicability of CW-MFCs as a **clogging assessment tool**.

OB (6)   To determine the **environmental and economic** impact of the implementation of MFCs in CWs.

CHAPTER 3

State of the art

3.1 Constructed wetlands

Constructed wetlands (CW) are natural wastewater treatment systems where wastewater is treated by means of physical, chemical and biological processes taking place within the treatment bed (García et al., 2010). They consist of shallow lined basins filled up with a filter media (generally gravel) and planted with aquatic plants such as macrophytes. CWs treat wastewater from a wide range of origins such as urban, industrial or agricultural wastewaters. They are also characterized by being low energy demanding systems and easy to operate and maintain. As a consequence, they have become an alternative to conventional intensified systems for the sanitation of small communities (García, 2001; Puigagut et al., 2007a).

Regarding wastewater flow characteristics, there are three main types of treatment wetlands (Kadlec and Wallace, 2009): Free water surface (FWS) wetlands, Vertical subsurface flow (VF) wetlands and Horizontal subsurface flow (HSSF) wetlands. The latter (HSSF), is the type of wetland in which this thesis will be focused and therefore, is described below in detail.

3.1.1 *Horizontal Subsurface constructed wetlands*

The CWs configuration most widely used is horizontal subsurface flow constructed wetlands. In HSSF CWs, water flows horizontally and below the surface of the granular medium. HSSF CWs are operated under saturated conditions and are, generally, shallower than other type of wetlands, with water depth being generally between 0.3 and 0.6 m. Removal rates of most of the contaminants in HSSF CWs are affected by design parameters such as the organic loading rate, the width to length aspect ratio, the granular medium size and the water depth (García et al., 2003). According to USEPA (2000) the main criteria for HSSF design is the organic loading. Accordingly, the loading rate should be 6 g BOD_5/m^2.day in order to produce an effluent with less than 30 mg/L BOD_5 day of inlet BOD. Moreover, Kadlec and Wallace (2009) defines an average needed surface of 5m²/PE to treat a primary-settled domestic wastewater.

Due to its anaerobic nature, HSSF CWs have relatively large surface requirements when compared to intensive technologies (such as activated

sludge-based treatment systems), which is one of its major drawbacks. Over the past years, research in HSSF CWs has focused on the improvement of treatment performances and the reduction of surface requirements. Among the strategies to increase CWs performances, forced (or active) aeration has been recently suggested as an efficient way to improve removal of organic matter and reduced nitrogen species (Austin and Nivala, 2009; Wu et al., 2014). Since the 1990s, active aerated systems have shown interesting results, leading to an increase of removal rates compared to passive systems (Nivala et al., 2013) resulting in the reduction of the required treatment surface. However, active aeration results in a significant increase in energy consumption during operation when compared to traditional HSSF CWs designs.

3.1.2 Contaminant removal processes

Although the main purpose of wastewater treatment processes is the elimination of organic matter, removal of other contaminants such as nitrogen or phosphorus is also of importance. Contaminant removal is carried out by means of physical, chemical and biological processes within the wetland. During the treatment of wastewater, the organic matter is degraded and nutrients such as nitrogen, phosphorous or sulphate are also removed. Therefore, organic matter removal in wetlands is simultaneously carried out by means of aerobic respiration, denitrification, sulphate reduction, fermentation or methanogenesis (García et al., 2010).

3.1.2.1 Organic matter

Organic matter includes dissolved and particulate components. Influent particulate organic matter is mainly retained by physical processes such as filtration and sedimentation. Retained particulate organic matter accumulates and undergoes a hydrolysis process, which generates dissolved organic compounds that can be further degraded by different biological pathways that occur simultaneously within the treatment bed. Redox conditions are very important in determining the extent and rate of contaminant removal rates (García et al., 2010; Maier, 2009). Accordingly, if conditions within the wetland are aerobic, oxygen will be used as the electron

acceptor and if they are anoxic or anaerobic, other electron acceptors (such as nitrate or sulphate) will be used leading to less efficient degradation pathways.

In HSSF CWs, where anaerobic conditions predominate, a very diverse microbial community grows within the system, and therefore, organic matter is degraded by many different pathways. In the first step of the degradation process, the hydrolysis of complex organic compounds such as carbohydrates or proteins produce simple compounds that are further hydrolysed to produce low chain fatty acids (ex. acetate). Acetate and other low chain fatty acids can be further degraded by organisms such as sulphate reducing bacteria or by methanogenic organisms. As a result of methanogeneis methane and CO_2 is produced.

3.1.2.2 Nitrogen

The main inorganic nitrogen compound in wetlands is ammonia (NH_4^+), the transformation of which ends up producing nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O) and dissolved elemental nitrogen or dinitrogen gas (N_2). In subsurface constructed wetlands nitrogen is mainly removed by means of plant uptake and nitrification and denitrification processes (García et al., 2010). However, depending on environmental conditions many other nitrogen removal pathways can occur within subsurface constructed wetlands such as anammox (Pelissari et al., 2016; Saeed and Sun, 2012).

While nitrification requires oxygen and low carbon contents, denitrification requires carbon and an anoxic/anaerobic environment. During nitrification, ammonia is oxidised to nitrite and nitrate under aerobic conditions. These two aerobic reactions are carried out by ammonium oxidizing bacteria which require 4.6 kg of oxygen per kg of NH_4^+-N to fulfil their metabolic reactions (Faulwetter et al., 2009). Moreover, the oxidation of ammonium is only developed at higher concentrations of dissolved oxygen (generally higher than 1 mg/L). To full-fill complete nitrogen removal denitrification must also take place within the treatment bed. Denitrification is carried out by facultative heterotrophic bacteria that are able to reduce nitrate or nitrite to nitrogen gas under anoxic conditions. Therefore, in constructed wetlands treatment plants, nitrogen is commonly eliminated by

two stage configurations able to reproduce required nitrification-denitrification conditions.

Moreover, nitrogen removal (both nitrification and denitrification) as a biological process, are affected by temperature changes, pH, loading rates or oxygen concentrations (Faulwetter et al., 2009). HSSF CWs are considered to be mainly anaerobic systems. Hence, nitrification processes which require oxygen to transform ammonia to nitrate are limited in this type of wetlands. However, several authors have reported total nitrogen removal rates such as 40 to 50% (Vymazal, 2007) or 33 to 68% (Puigagut et al., 2007b). Caselles-Osorio and García (2007) stated that shallow HSSF CWs can achieve an ammonium removal higher than an 80%.

3.1.3   Redox state

The main mechanisms by which the microorganisms degrade organic matter are aerobic respiration and fermentation. The metabolic pathway for organic matter oxidation depends on the final electron acceptor available and, therefore, on the oxidation-reduction conditions within the wetland (Faulwetter et al., 2009). Redox conditions influence not only the chemical but also the microbiological reactions (Kadlec and Wallace, 2009). As explained in Kadlec and Wallace (2009) the oxidation of the organic matter provides energy to bacteria. The amount of energy depends on the nature of the electron donor and acceptor. The most efficient electron acceptor is oxygen (O2). Only after oxygen is depleted or not available other electron acceptors (such as NO_3^-, MNO_2, $FeOOH$, SO_4^{2-} and finally CO_2) will be used for OM oxidation. Accordingly, the degradation pathways follow a sequence depending on redox potentials (Table 3.1) (Faulwetter et al., 2009).

Table 3.1 Selected types of microbial redox reactions in constructed wetlands. (Faulwetter et al., 2009)

Process	Electron acceptor	End products	Redox potential
-	-	-	mV
Aerobic respiration	O_2	H_2O	300 to 700
Nitrate reduction	NO_3^-	N_2, NO_x	100 to 350
Manganese reduction	Mn_4+	Mn_2^+	-100 to 300
Iron reduction	Fe_3^+	Fe_2^+	-100 to 200
Sulphate reduction	SO_4^{2-}	S_2^-	-200 to -100
Methanogenesis	CO_2	CH_4 , CO_2	-350 to -100

Redox conditions in HSSF CW are variable and depend on several factors. To this regard, it is described that there are both oxidized and reduced zones within the wetland at the same time. This type of systems, however, is considered to be mainly anaerobic (Baptista et al., 2003). The factors that can influence the redox conditions in horizontal subsurface flow wetlands have been assessed by some authors. García et al. (2003) determines that redox potential decreases with depth and increases with length. The explanation given for the first is that the oxygen diffusion from the atmosphere and the oxygen plant supply through the roots are processes being carried out at the upper layers. The increase with length is due to the progressive oxidation of the organic matter along the treatment bed which leads to higher oxidized conditions close to the outlet of the treatment bed (García et al., 2003).

Moreover, some authors have also measured redox potentials within the wetland. García et al. (2003) found values from -200 to 200 in HSSF beds. Dušek et al. (2008) found fluctuations of the redox potential from -400 to 800 mV in an experiment of 2 years. These fluctuations were higher in the upper layer and closer to the effluent.

Other operational parameters such as plants (Dušek et al., 2008), the HRT (Faulwetter et al., 2009) or the organic matter concentration (Kadlec et al., 2000) also influence redox potential within the wetlands. CWs can be designed to foster a wide range of redox conditions to remove a higher variety of pollutants in the same bed or to foster some of the removal pathways by considering all the parameters defined.

3.1.4 *Microbial wetlands' community*

In CWs, nutrient removal is mostly carried out by means of microbial degradation which includes, in many cases, several steps and organisms which are interconnected (Kadlec and Wallace, 2009). This degradation, or biodegradation, is defined as the breakdown of the organic compounds due to respiration and fermentation carried out by microorganisms (Faulwetter et al., 2009; Maier, 2009). During the degradation process, each degradation step is catalysed by a specific enzyme made by the degrading cell. Moreover, most bacteria use the substrate contained in the surrounding aqueous phase (Maier, 2009). However, those organic compounds with low water solubility must be degraded when in contact with the microorganisms and thus, its degradation is limited.

Constructed wetlands have a bacterial community which remains relatively stable in time (Truu et al., 2009). However, microbial activity can be affected by physical and chemical conditions and also biological factors. In fact, under certain conditions, there is a maximum level of microbial activity that can be recorded (Maier, 2009). Filter material, hydraulic conditions, the presence of plants, the temperature or the organic loading are among the main factors influencing the microbial community activity (Baptista et al., 2003; Maier, 2009; Nguyen, 2000; Truu et al., 2009). Accordingly, Truu et al. (2009) described spatial differences in microorganisms established regarding the depth and the distance to the inlet. Baptista et al. (2003) found differences between planted and unplanted systems regarding the microbial community and Gagnon et al. (2007) report higher microbial densities in the rhizosphere regions.

3.1.5 Presence of plants

As stated in Kadlec and Wallace (2009) macrophytes are an important element to achieve a high-quality treated water in wetland systems. Actually, direct pollutant immobilization is important during the plant growth. However, this plant uptake is not commonly the main removal process in constructed wetlands if compared to microbial or physical reactions (García et al., 2010; Kadlec and Wallace, 2009). Caselles-Osorio and García (2007) reported that more oxidised conditions and higher removal rates were found in wetlands with active aboveground biomass (leaves and stems).

The contribution of plants in the treatment process is due to direct plant uptake but also due to the fact that macrophytes are able to transfer oxygen to the rhizhosphere (Brix, 1997). This oxygen is released by plants in order to survive within the reduced environment existing in wetlands. Stottmeister et al. (2003) describes the formation of an oxidative protective biofilm on the root surface of a thickness ranging between 1 and 4 mm which includes the potential difference that exist between the rizhosphere (reduced environment) and the root surface (oxidized environment). The release of oxygen from the plants depends on factors such as the redox potential (Faulwetter et al., 2009) or the plant species (Brix, 1997; Stottmeister et al., 2003).

Carbon compounds are also released to wetlands from plants by a process known as rhizodeposition. These organic compounds, such as sugars or amino acids, have a very diverse composition. Although these carbonaceous compounds can be used by bacteria as substrates, the relative low amount of organic matter introduced to the system would be only significant in very low loaded systems (Stottmeister et al., 2003).

Vegetation has also been described to exert a great impact on CWs redox conditions by means of evapotranspiration (Pedescoll et al., 2013a). Evapotranspiration is described by Kadlec and Wallace (2009) as water losses from a wetland to the atmosphere that can be divided between evaporation (from water and soil) and transpiration (from the aboveground plant biomass). Furthermore, evapotranspiration is described to induce changes in the water table (Mann and Wetzel, 1999) and therefore, has an influence on

redox conditions within the wetland (Pedescoll et al., 2013a). Pedescoll et al. (2013a) found a positive relationship between evapotranspiration and redox conditions in HSSF CWs due to fluctuations in water table that promoted natural aeration of the filter medium and as a consequence of increased oxidized conditions in the upper layer.

3.1.6   Clogging phenomena

HSSF CWs are subjected to a progressive reduction of their hydraulic conductivity and porosity. This process is generally known as clogging. Clogging of HSSF CWs occurs due to the mechanisms described below (Kadlec and Wallace, 2009; Knowles et al., 2011):

- Deposition of inert (mineral) suspended solids in the inlet region of the wetland bed

- Accumulation of refractory organic material (resistant to microbial degradation) in the inlet zone of the wetland bed

- Deposition of chemical precipitates in the wetland bed

- Loading of organic matter (both suspended and dissolved) that stimulates the growth of microbial biofilms on the bed media

- Development of plant root networks that occupy pore volume within the wetland bed

Most of the accumulated solids within the filter media have a low proportion of organic matter (Caselles-Osorio et al., 2007; Knowles et al., 2011; Pedescoll et al., 2011a). To this regard, Caselles-Osorio et al. (2007) states that the content of organic matter in clogged media is less than 20% and that settleable and macrocoloidal influent COD fraction is the most accumulated fraction. Also Pedescoll et al. (2011a) reports that volatile solids represented less than the 50% on total solids regardless the operational conditions of the wetlands studied. Moreover, and after aerobic and anaerobic biodegradability tests, Caselles-Osorio et al. (2007) determined that the organic matter accumulated was not easily biodegraded.

Clogging is, at least partially, a consequence of solids accumulation. Moreover, the composition and the quantity of accumulated solids depend not only on the load applied to the CWs (Knowles et al., 2011) but also on other environmental conditions. To this regard, Caselles-Osorio et al. (2007) states a positive relationship between the quantity of solids accumulated and both TSS and COD loading rates. Pedescoll et al. (2011a) implemented two different primary treatments (HUSB reactor vs. conventional settlers) and found higher accumulations of solids in the wetlands fed by the anaerobic treatment. The latter also described an important contribution to clogging of plants root system (between a 10% and a 40% of reduction in the hydraulic conductivity was found in planted systems when compared to unplanted ones).

Clogging is not a homogeneous phenomenon along the length of the wetland. Several authors have described greater solids accumulation in the inlet zone due to higher organic matter concentrations (Knowles et al., 2011). Other authors have also stated a higher reduction of hydraulic conductivity at the inlet zone of wetlands (Caselles-Osorio et al., 2007; Kadlec and Wallace, 2009). Clogging is not only taking place differently along the length of the wetlands but also along the depth. Accordingly, Pedescoll et al. (2011a) reported higher rates of sludge accumulation at the bottom of the wetlands.

Finally, bed clogging is the cause of most of the operational wetlands' problems (Kadlec and Wallace, 2009). To solve clogging two different management strategies have been proposed in the literature: preventive strategies and restorative strategies (Nivala et al., 2012). Intermittent operation, multiple influent distribution and minimization of the inlet cross-sectional loading would be some of the preventive ones, while excavation and replacement of the gravel, washing and reuse of the gravel or application of chemicals would constitute restorative strategies.

However, addressing the management of the clogging leads to an increase in maintenance costs in HSSF CWs. In fact, it is assumed that inlet zone maintenance, conducted every 5 years, may account for up to 15% of construction costs (Kadlec and Wallace, 2009). Therefore, finding a cost-effective solution to clogging phenomena is of capital importance for

increasing the lifespan of HSSF CWs and improving the economic management (Kadlec and Wallace, 2009).

3.2 Microbial Fuel Cells

3.2.1 Description of the technology

Microbial Fuel Cells (MFCs) are bioelectrochemical systems that generate current by means of electrochemically active microorganisms as catalysts (Logan et al., 2006). In a MFC, organic and inorganic substrates are oxidized by bacteria and the electrons are transferred to the anode from where they flow through a conductive material and a resistor to a higher redox electron acceptor, such as oxygen, at the cathode (Logan et al., 2006; Rabaey et al., 2007) (Figure 3.1).
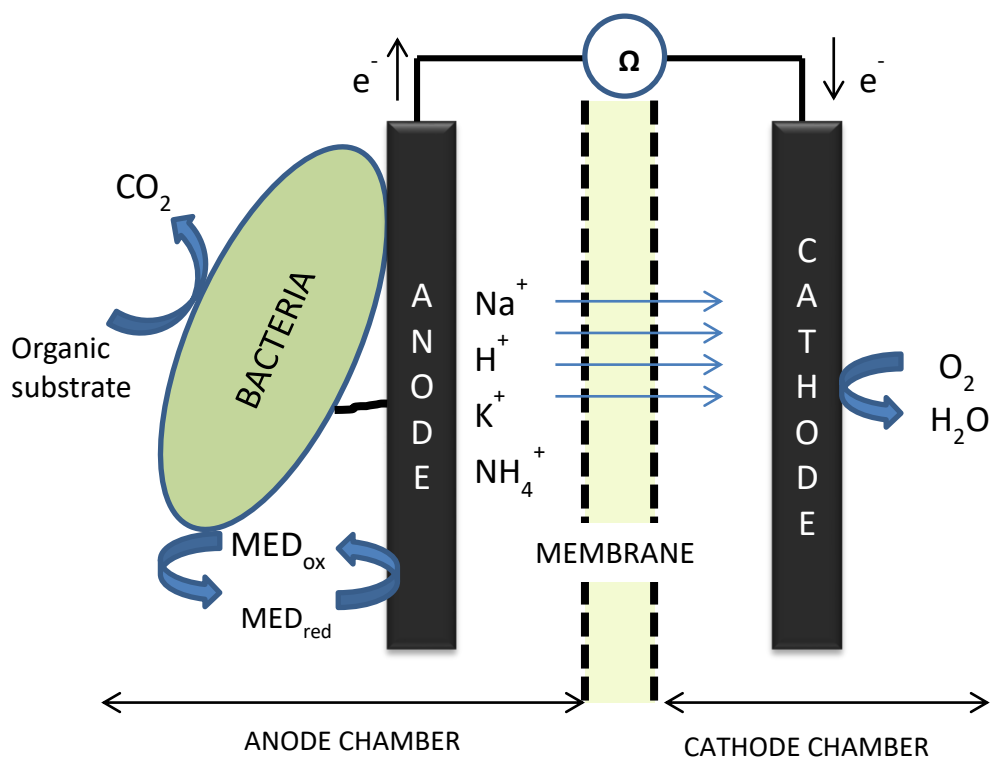
Figure 3.1 Scheme of a microbial fuel cell (MFC) and its main processes.

There are two well-known bacterial genera which present exoelectrogenic activity in pure culture, i.e., *Shewanella* (Ringeisen et al., 2006)

and *Geobacter* (Kiely et al., 2011; Richter et al., 2008). To date, a high diversity of microorganisms has been described to perform anode respiration to a certain degree (Logan, 2009). Over 20 different exoelectrogenic bacteria have been reported in the last decade, belonging to diverse phylogenetic groups (Logan, 2009). However, the power density achieved in most of the experiments working with mixed cultures is higher than in pure cultures (Nevin et al., 2008; Rabaey and Verstraete, 2005). These results reinforce the idea that increased electricity generation could be attributed to synergistic interactions within the microbial community. Namely, there could be microorganisms that do not exchange directly electrons with the electrode, but could be setting up interactions among other members of the microbial community playing a crucial role in the operation of a MFC. In fact, recent studies have reported the presence of archaeal cells, such as *Methanosaeta* and *Methanosarcina*, attached to the biofilm in the anode of MFCs, suggesting that they might play a role promoting syntrophic interactions with exoelectrogenic eubacteria in the anode biofilm (Chung and Okabe, 2009; Rotaru et al., 2014a, 2014b; Sotres et al., 2015).

Compounds oxidized at the anode are mainly simple carbohydrates such as glucose or acetate that can be already present in the environment or obtained from the microbial degradation of complex organic substrates such as organic sediments or wastewater (Min and Logan, 2004; Reimers et al., 2001). MFCs are, therefore, an alternative technology to harvest energy directly from wastewater in the form of electricity (Du et al., 2007; Lefebvre et al., 2011; Min and Logan, 2004). In order to ensure the use of the anode as the final electron acceptor by electrochemical active microorganisms, no acceptor with higher redox potential shall be present in their vicinity (Lefebvre et al., 2011). Consequently, the electromotive force (E_{emf}) of the cell will depend on the potential of the anode (E_{an}) and therefore, on the redox gradient between the anode and the cathode (Logan et al., 2006; Rabaey et al., 2007). Finally, the measured cell voltage is the electromotive force minus the overpotentials (both at the anode (η_a) and at the cathode (η_c)) and the ohmic losses of the system (Logan et al., 2006) (see Eq. 1 and 2).

$$E_{emf} = E_{cat} - E_{an} \quad \text{(Eq. 3.1)}$$

$$E_{cell} = E_{emf} - \left(\sum \eta_a + \left| \sum \eta_c \right| + IR_\Omega \right) \qquad \text{(Eq. 3.2)}$$

In order to provide a redox gradient between the anode and the cathode of an MFC, two different strategies may be applied. The first strategy is to use a proton exchange membrane (PEM) between the electrodes that enables the existence of an electromotive force between the electrodes by only allowing the transfer of charges between the anode and the cathode zones. Another strategy is to exploit the natural redox gradient existing between surface waters and organic sediments in natural or semi-natural environments. The later MFC design is generally known as sediment or benthic microbial fuel cell (sMFC). Implementing a PEM between the electrodes allows us to have a greater cell force between electrodes, yet it results in a more expensive set up (of difficult scalability) when compared to MFC operated without a PEM (sMFC configuration).

3.2.2 *MFCs' performance influencing factors*

MFCs' performance is influenced by several factors, including biological, chemical and also electrical factors. These factors are widely described and optimized in literature. Rabaey and Verstraete (2005) divides the parameters defining MFCs' performance in: 1) Substrate conversion rate; 2) Overpotentials at the anode; 3) Overpotentials at the cathode; 4) Proton exchange membrane related factors and 5) Internal resistance of the MFC. However, operational parameters such as the concentration of COD in the anodic chamber, PH or the temperature together with the surface area of electrodes, its materials and its relative distance have also been reported as influencing factors (Jadhav and Ghangrekar, 2009).

Some of these factors, such as the electrodes' materials, the electrodes' relative distance, electrodes' relative areas, concentration of OM within the anodic compartment or the availability of oxygen at the cathodic compartment are described in more detail below as they are considered of capital importance for the purpose of this thesis.

3.2.2.1   Electrode materials

Maximizing MFCs power generation and its coulombic efficiency by determining optimal materials is reported as one of the challenges in the field of MFCs (Logan, 2008). Besides, these materials are required to be sustainable and structurally stronger in order to implement the technology in full-scale facilities (Logan, 2008).

Anodic materials should be conductive and non-corrosive, have high specific surface areas (area per volume) and high porosity, should be non-fouling, inexpensive, easily made and scalable to larger sizes (Logan, 2008). Moreover, they should be impervious to biological or abiotic corrosion, passivation or degradation (Girguis et al., 2010) but, at the same time, bacteria must be able to grow at the material's surface and allow a high rate electron transfer (Logan, 2008). Carbon is described as the most versatile electrode material due to the fact that it can be found in market as rods or granules, compact graphite plates, fibrous material (felt, cloth, paper, fibers, foam) or glassy carbon (Logan et al., 2006). Also graphite sheet, plates and rods have been commonly used as anodes because their material strength, inertness and commercial availability (Girguis et al., 2010). However, some authors have also assessed the use of non-carbon materials such as gold or stainless steel (Zhou et al., 2011).

Cathode functions as the electron donor to reduce an electron acceptor present in the cathodic compartment. Cathode's design has been described as the greatest challenge to scale up the technology (Logan, 2008). This challenge comes from the fact that reactions taking place at the cathode are difficult to engineer (Logan, 2008). Therefore, cathode material has a great impact on MFCs performance (Logan et al., 2006; Zhou et al., 2011).

The materials used for the cathode are generally the same than the ones described for the anode (Girguis et al., 2010). To this regard, graphite, carbon cloth or carbon paper are commonly used materials to build cathodic electrodes (Zhou et al., 2011). However, when these materials are used as cathodic materials, they are usually modified with a catalyst (Logan, 2008). Ferricyanide or Pt are commonly used as catalysts (Logan et al., 2006).

3.2.2.2 Electrode area and distance between electrodes

Anodic and cathodic specific areas are a determining parameter in order to maximize MFCs production. Although not all electrodes' surface is available for the bacteria to grow, power generated is dependent on the relative surface areas between the anode and the cathode (Logan et al., 2006).

Oh and Logan (2006) assessed the effect of increasing the cathode surface area relative to that of the anode and found that for the first increase (by a 175%) power was increased by 200% of the while for the second increase (by a 350%) the power only increased by a 58%. The explanation was that the proportionality between the cathode surface and the power output was only followed when no other factor was limiting (such as PEM surface). Actually, an equation to describe the proportional relation was reported in which the power output may be predicted as function of the electrode and PEM surfaces (Oh and Logan, 2006). Furthermore, Ghangrekar and Shinde (2007) studied the influence of increasing the anode and found that as the anodic area was increased, the power density was decreased, indicating that the anode was used less efficiently when its surface was increased.

The distance between electrodes has also been described as one of the factors adding resistance to the system and, as a consequence, having an influence on MFCs power production. Ghangrekar and Shinde (2007) assessed the electrical output testing three different distances between the anode and the cathode (20, 24 and 28 cm) and determined the higher the distance between electrodes the lower the power density recorded.

3.2.2.3 Anodic reactions

MFCs' power output is directly linked to the organic matter present in the anodic chamber, so the higher the amount of organic matter the higher the amount of fuel for exoelectrogenic bacteria to oxidise. Organic substrates used to operate microbial fuel cells can be very complex in composition (i.e wastewater). Therefore, an hydrolysis process where fatty acids, aromatic compounds, fermentable sugars and amino acids are produced must take place, before exoelectrogens can oxidize them (Lovley, 2006). The hydrolysis

process will end up producing compounds such as acetate or lactate that support the growth and activity of extracellular electron transfer bacteria.

Gil et al. (2003), Gonzalez del Campo et al. (2013), Jadhav and Ghangrekar (2009), Juang et al. (2011) and Rodrigo et al. (2007) found a relationship between the organic matter content and MFCs performance. However, while Gil et al. (2003), Gonzalez del Campo et al. (2013), Juang et al. (2011) and Rodrigo et al. (2007) reported higher currents when higher organic matter concentrations were provided, Jadhav and Ghangrekar (2009) found a linear relationship between the current output and the COD removal in the anodic chamber. Juang et al. (2011) also reported a concomitant increase in power densities and COD degradation rates. Moreover, Rodrigo et al. (2007) quantified, taking into account the oxygen consumed at the cathode, that only a 0.25% of the influent COD removal was used for electricity generation and, therefore, that the majority of the COD was removed by anaerobic processes. Also the influence of the flow rate was studied in Rodrigo et al. (2007) and, on the contrary to organic matter concentration, no relation was found between the flow rate and the electrical output. In the paper it is suggested that increasing the flow rate did not improve mass-transfer coefficient. The ability of MFC to respond accordingly to organic matter concentration has resulted in their use a biosensor tool for organic matter assessment (Gil et al., 2003). To this regard, MFC have been used as biosensors to detect lactate, fructose and the total organic strength of wastewater in terms of biological oxygen demand (BOD) (Chang et al., 2004).

3.2.2.4 Cathodic reactions

The availability of the electron acceptors at the cathode is necessary to close the electrical circuit and the redox reaction. Although several chemical electron acceptors such as ferricyanide or permanganate (Logan and Regan, 2006; You et al., 2006) have been used in the cathodic chamber, efforts must be focused on optimizing oxygen-MFCs. Oxygen has a high oxidation potential, availability, low cost, sustainability and lack of chemical waste products and therefore, it is the most suitable electron acceptor to be used (Logan et al., 2006). To provide oxygen to the cathode, two different strategies are possible: direct air or soluble oxygen in water. Although oxygen can diffuse into water, its solubility (mole fraction basis) is only of 4.6·10-6 (25ºC)

compared to 0.21 in air (Logan, 2008) and that is why mainly air-cathode MFCs are being developed. However, oxygen reduction at non-catalysed materials cause large overpotentials (Rabaey et al., 2008).

Overall, cathodes have been widely described to be the main limiting factor of MFCs' performance in several papers (Ahn et al., 2014; Fan et al., 2008). Power generation has been demonstrated to be dependent on oxygen availability at the cathode (Ahn et al., 2014; Ferreira-Aparicio and Chaparro, 2014; Oh et al., 2004; Zhang et al., 2013) and it has been also proven that air cathodes, compared to aqueous ones, lead to higher cell output not only due to higher oxygen concentrations but also to higher mass transfer rates (Fan et al., 2008). Furthermore, in conventional MFCs, cathode flooding (Ferreira-Aparicio and Chaparro, 2014; Zhang et al., 2013), air humidity and water pressure (Ahn et al., 2014) have been described to be factors affecting oxygen accessibility. Moreover, the cathode as the power limitation has also been reported in a plant-MFC (Strik et al., 2008).

3.3 Microbial Fuel Cells implemented in Constructed wetlands

In some real aquatic environments exist natural redox gradients that can be exploited to produce energy via MFC implementation. So far, MFC have been mostly implemented in rice paddy fields (De Schamphelaire et al., 2008; Kaku et al., 2008), marine sediments (Reimers et al., 2001; Rezaei et al., 2007) or planted systems (Strik et al., 2008; Venkata Mohan et al., 2011). Furthermore, horizontal subsurface flow constructed wetlands (HSSF CWs) are engineered systems used for wastewater treatment that are subjected to great spatial redox variations (especially in depth) (García et al., 2003). Although the system is mainly anaerobic (Baptista et al., 2003), the very upper part of the wetland remains under aerobic conditions because its close contact with the atmosphere giving redox gradients of about 0.5 V vs SHE (Dušek et al., 2008; García et al., 2003; Pedescoll et al., 2013a). As a result, natural redox gradients in HSSF CWs could be exploited to produce energy via MFC implementation.

Due to the nature of both technologies, MFC and CW, the synergy between them becomes possible. The feasibility of the implementation of MFCs in CWs is based on the presence of organic matter (OM) in the system

which comes from wastewater but also because there is a naturally generated redox gradient between the upper zone (in contact with the atmosphere and therefore in aerobic conditions) and the deeper zone (in completely anaerobic conditions) of the treatment bed (García et al., 2003). CW-MFCs electricity production would be of special interest in regards to CWs, since one of the major advantages of this technology is the low energy input necessary for wastewater treatment (<0.1 kWh·m^{-3})(Kadlec and Wallace, 2009). Beyond the generation of electricity, the implementation of MFCs in CWs can have several potential environmental applications. The improvement of CWs treatment efficiency or the organic matter and clogging bioindication are some of them.

Recently the implementation of MFCs in CWs has been also reported (Fang et al., 2015; Villaseñor et al., 2013; Yadav et al., 2012). The application of the technology in full-scale conditions leads to issues regarding the complexity of the environment which is often not controllable. Nowadays, CW-MFCs constitute a trending topic being investigated for several research groups. Although here were only few reported investigations at the beginning of this thesis, multiple articles have been published about the topic during the last 5 years (Figure 3.2). Therefore, this chapter aims on including published knowledge about the synergy between technologies, in order to introduce the objectives of this thesis.
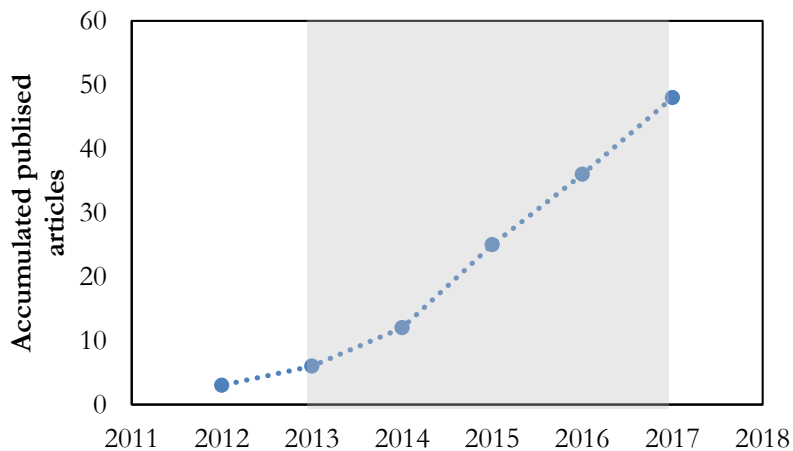
Figure 3.2 Published articles on the topic: "constructed wetland" and "microbial fuel cell". Thesis development is marked in grey. Note: data has been collected by using Scopus at 16/06/2017.

3.3.1 Previous work: architecture and operation of CW-MFCs

Despite the inherent variability of the reported set-up designs and the operational conditions in which experiments were conducted, there are some common characteristics. Table 3.2 shows a summary of the most important parameters defining the set-up and the operation of CW-MFCs. The majority of these experiments did not use real wastewater and most of them were based on the application of lab-scale systems simulating constructed wetlands. Only one author described the implementation in a pilot-scale constructed wetland (Villaseñor et al., 2013). Both the usage of synthetic wastewater and the laboratory scale, guarantee constant and controllable conditions along the experimental period but diverge from the reality of a real-scale constructed wetland treatment plant. Also all the systems reported were planted and different species were used: Canna indica, Ipomoea aquatic, Phragmites australis or Elodea nuttallii (Fang et al., 2013; Lu et al., 2015; Oon et al., 2017; Villaseñor et al., 2013). Presence of plants in CW-MFCs can have different effects on systems performance. The influence of plants on CW-MFC performance has been also currently addressed (Oon et al., 2017). Plants influence CW performance due to their ability to release oxygen or easily

biodegradable substrates through the root system. Therefore, CW-MFCs set-up can be designed to place the rhizosphere in the cathodic area to foster the presence of oxygen, but also within the anodic area assuming that the exudates are oxidized to produce energy (Xu et al., 2016). Accordingly, rizhodeposits are reported to be a viable sole electron donor thus producing electricity without any other nutrient addition (Lu et al., 2015). Also the presence of plants has been reported to stimulate the microbial diversity (Lu et al., 2015) and to produce higher currents (Fang et al., 2013) when compared to non-planted systems. In the context of constructed wetlands, plants are widely described to participate on the evapotranspiration which can affect redox conditions within the systems (Pedescoll et al., 2013a). Presence of plants in CW-MFCs are reported to change redox conditions within the cathodic area thus causing a fluctuation in the current due to the light/darkness cycle of the photosynthetic activity (Liu et al., 2013; Villaseñor et al., 2013).

Redox conditions within the system are also determined by the operation flow strategy (Doherty et al., 2015). As it is clearly seen in Table 3.2, the majority of investigations used upflow devices. By means of this arrangement, wastewater enters the system at the bottom and flows firstly trough the anodic volume where there are anaerobic conditions and organic matter is oxidized by exoelectrogenic bacteria and then, through the cathodic volume, were heterotrophic bacteria uses oxygen as the final electron acceptor to oxidize the remaining organic compounds. This type of systems, although not reproducing any real-scale constructed wetland, are linked to very high OM removal rates as a consequence of the two different redox conditions to which wastewater is subjected (anaerobic and aerobic).

The concentration of oxygen at the cathode is a crucial factor for MFCs performance (Ahn et al., 2014). Constructed Wetlands are a natural based wastewater treatment technology. Accordingly, when considering a CW-MFCs, apart from the energy generated by the system itself, the energy consumption must be minimized. Therefore, as seen in Table 3.2, most of the investigations used an air-cathode. In air-cathode systems, oxygen works as the final electron acceptor and comes directly from the atmosphere (Liu et al., 2013). Also other configurations like forced aeration, rhizosphere cathode or slightly submerged cathode have been reported. Although forced aeration at the cathodic area significantly improves organic matter removal efficiencies of

the system, it also causes operational costs (Zhao et al., 2013). However, some authors support that the aeration flow rate can be adjusted to an optimum value at which OM, nitrate and ammonium removal rates are maximized and there is also energy recovery with MFCs (Oon et al., 2017).

The effect of the external resistance on system's performance have been widely studied in conventional MFCs (Jadhav and Ghangrekar, 2009; Katuri et al., 2011). When this technology was implemented in constructed wetlands, the majority of the studies used resistances around 1000 ohms. Furthermore, some studies calculated the internal resistances of the systems. Due to the complexity of these type of systems, internal resistances are known to be significantly higher than in conventional MFCs. Reported internal resistances in CW-MFCs regardless their architechture and operation are between 120 to 1471 Ω (Table 3.2).

Graphite and carbon are commonly used as electrode materials in CW-MFCs (Table 3.3). Long term stability, good surface for microbial communities to establish and high electrical conductivity are some of their properties (Doherty et al., 2015). In conventional MFCs modifications of the electrode materials are widely used. For example, MFCs operated with real wastewater are commonly operated with a cathode catalyst normally containing Pt (Zhou et al., 2011). On the contrary, in the implementation of MFCs in constructed wetlands always non-altered materials are used, like graphite or granular activated carbon (GAC) which is coherent with the natural treatment line of constructed wetlands. However, as depicted in Table 3.3, different material configurations are used such as, for example, plate, granules, rod or felt for graphite.

Table 3.2.A Summary of architecture and operational conditions of CW-MFCs

Reference	Reactor volume (L)	Planted /Unplanted	Flow direction	Carbon source	Oxygen source	External Resistance (Ω)	Internal Resistance (Ω)
Yadav et al. (2012)	2.3 / 5	planted	Batch	Synthetic WW	air-cathode	-	-
Zhao et al. (2013)	3.7 / -	planted	Batch/ Upflow	Swine WW	forced aeration	12000	-
Liu et al. (2013)	12.4 / 35.3	planted	Upflow	Synthetic WW	air-cathode	1000	156-256
Villaseñor et al. (2013)	108 / 270	planted	Horizontal flow	Synthetic WW	12 cm submerged	120	120
Fang et al. (2013)	12.4 / 35.3	planted	Upflow	Synthetic WW	air-cathode	1000	218-273
Liu et al. (2014)	1.4 / 3.9	planted	Upflow	Synthetic WW	air-cathode	1000	209-582
Lu et al. (2015)	- / 2.65	planted	Batch	Rizhodeposits	air-cathode	1000	-
Oon et al. (2015)	- / 19	planted	Upflow	Synthetic WW	forced aeration	1000	820-4300
Doherty et al. (2015)	8.1 / -	planted	Upflow-Downflow	Swine WW	-	950	500-300
Srivastava et al. (2015)	1.8 / -	planted	Batch	Synthetic WW	air-cathode	-	-
Fang et al. (2015)	- / 35.3	planted	Upflow	Synthetic WW	air-cathode	1000	-
Oon et al. (2016)	- / 19	planted	Upflow	Synthetic WW	forced aeration	1000	200-430

Table 3.2.B Summary of architecture and operational conditions of CW-MFCs

Reference	Reactor volume (L)	Planted /Unplanted	Flow direction	Carbon source	Oxygen source	External Resistance (Ω)	Internal Resistance (Ω)
Xu et al. (2016)	1.43 / 4.08	planted	Upflow	Synthetic WW	air-cathode, rizosphere cathode	500	188-319
Wang et al. (2016)	- / 4.08	planted	Batch	Synthetic WW	air-cathode	1000	-
Wang et al. (2016)	- / 10.46	planted	Batch	Synthetic WW	air-cathode	1000	-
Fang et al. (2016)	- / 19	planted	Upflow	Synthetic WW	air-cathode	1000	632-1471
Oon et al. (2017)	- / 11.3	planted	Upflow	Synthetic WW	forced aeration	1000	200-450
Fang et al. (2017)	12.4 / 35.3	planted	Upflow	Synthetic WW	air-cathode	1000	652-1471
Song et al. (2017)	2 / 5	planted	Upflow	Synthetic WW	air-cathode	-	340-406
Xu et al. (2017)	1.43 / 4.08	planted	Upflow	Synthetic WW	air-cathode	500	130
Wang et al. (2017)	1.43 / 4.08	planted	Batch	Synthetic WW	air-cathode	1000	-
Note: Reactor volume is expressed in liquid volume/total reactor volume							

Table 3.3 Summary of the anode and cathode materials used in CW-MFCs

REFERENCE	ANODE	CATHODE
Zhao et al. (2013)	Graphite (plate)	Graphite (plate)
Liu et al. (2013)	GAC	GAC+SSM
Villaseñor et al. (2013)	Graphite (rectangular)	Graphite (rectangular)
Fang et al. (2013)	GAC	GAC+SSM
Liu et al. (2014)	GAC	GAC+SSM
Lu et al. (2015)	Graphite disk	Carbon cloth
Oon et al. (2015)	Carbon felt	Carbon felt
Doherty et al. (2015)	Graphite (granular)	Graphite (granular)
Srivastava et al. (2015)	Graphite (granular), GA charcoal	Graphite (granular), GA charcoal
Fang et al. (2015)	GAC+SSM	GAC+SSM
Oon et al. (2016)	GAC	GAC
Xu et al. (2016)	GAC	GAC / Graphite (felt)
Wang et al. (2016)	Carbon fiber felt, SSM, Graphite (rod), Foam nickel	Carbon fiber felt, SSM, Graphite (rod), Foam nickel
Wang et al. (2016)	Carbon fiber felt	Carbon fiber felt
Fang et al. (2016)	GAC	GAC
Oon et al. (2017)	Activated carbon	Activated carbon
Fang et al. (2017)	GAC	GAC
Song et al. (2017)	GAC	GAC
Xu et al. (2017)	Graphite (gravel) + SSM	Graphite (gravel) + SSM
Wang et al. (2017)	Carbon fiber felt, SSM, Graphite (rod), Foam nickel	Carbon fiber felt, SSM, Graphite (rod), Foam nickel
Note: GAC (granular activated carbon); SSM (stainless steel mesh); GA (granular activated)		

3.3.2 CW-MFCs for electricity generation

One of main benefits linked with the synergy between MFCs and CWs is the generation of electricity at the time wastewater is treated. MFCs convert the chemical energy contained in wastewater organic substrates to electrical energy which can be harvested and used within the treatment plant.

Although conventional MFCs operated with wastewater are able to produce electricity up to 12 W·m-3 (Logan and Rabaey, 2013), CW-MFCs are systems subjected to high internal resistances and therefore, the reported electrical production rates are much lower than the ones reported for conventional MFCs. Although the highest electrical output from CW-MFCs reported was of 2 W·m-3 (Xu et al., 2017) the average value of the studies considered was of 0.45±0.59 W·m-3 (Table 3.4). Focusing on the electricity produced by the systems operated with real wastewater, both of them are operated with swine slurry and the electrical productions reported were of 9.4 mW·m-2 (Zhao et al., 2013) and 276 mW·m-3 (Doherty et al., 2014).

In CW-MFCs, redox gradient between electrodes and therefore, the voltage produced, is naturally generated. No external currents are applied to impose certain redox potentials between electrodes neither specific reductants or oxidants are added to guarantee anaerobic or aerobic conditions at surroundings of electrodes. Accordingly, voltages produced by CW-MFCs are very variable thus they are dependent on the set-up configuration and the wastewater used for the operation, most of which is synthetic wastewater (Table 3.4). More into detail, voltages reported ranged between 117±21 mV (average value) (Wang et al., 2016) and 1161 mV (max value) (Villaseñor et al., 2013).

Also due to the nature of wastewater, which is composed by complex organic substrates, CE reported for CW-MFCs are significantly lower than for conventional MFCs. Pure culture studies inoculated with *Geobacter sulfurreducens* and acetate as the electron donor showed CE of 80±3% (Speers and Reguera, 2012). In CW-MFCs in which there is a very diverse anodic community and also electron and carbon source, CE reported ranged from 0.10% (Zhao et al., 2013) to 10.28% (Oon et al., 2017), indicating that most of the organic matter is removed by means of alternative oxidation pathways.

Table 3.4 Summary of operational conditions of electrical output and COD removal efficiencies of CW-MFCs.

Reference	Vol (L)	Power	Voltage	Coul. Eff	COD RE
Yadav et al. (2012)	2.3 / 5	15.73 mW/m^2	-	-	75%
Zhao et al. (2013)	3.7 / -	9.4mW/ m^2	371+-79 mV (average)	0.10 %	71.5-76.5 %
Liu et al. (2013)	12.4 / 35.3	12.42 W/ m^2	640mV (average)	-	94.80 %
Villaseñor et al. (2013)	108 / 270	43 mW/ m^2	1161 mV (maximum)	0.45 %	90-95 %
Fang et al. (2013)	12.4 / 35.3	302mW/m3	610 mV (maximum)	-	85.65 %
Liu et al. (2014)	1.4 / 3.9	44.63 mW/ m^2	525.3+-5.2 mV (av.peak)	8.86 %	89.90 %
Lu et al. (2015)	- / 2.65	18 mW/ m^2	-	-	-
Oon et al. (2015)	- / 19	6.12mW/ m^2	422 mV (maximum)	8.86 %	100 %
Doherty et al. (2015)	8.1 / -	276 mW/m^3	434+-14 (average)	0.36 %	81 %
Srivastava et al. (2015)	1.8 / -	320.8 mW7 m^3	760 mV (maximum)	-	81-91 %
Fang et al. (2015)	- / 35.3	852 mW/ m^3	667mV (maximum)	1.89 %	85.66 %
Oon et al. (2016)	- / 19	93mW/ m^3	421+-16mV (maximum)	1.42 %	99 %
Xu et al. (2016)	- / 14	10.77+-0.52 mW/ m^2	329+-16 mV (average)	2.12 %	-
Wang et al. (2016)	1.43 / 4.08	-	177 mV (max average)	-	52.30 %
Wang et al. (2016)	- / 4.08	8.08 mW/ m^2	117+-21 mV (average)	0.01-0.11 ‰	44.5 %
Fang et al. (2016)	- / 10.46	80 mW/ m^3	246 mV+-45 (average)	-	90 %
Oon et al. (2017)	- / 19	184.75 mW/ m^3	545.77+-25 mV (average)	10.28 %	99.00 %
Fang et al. (2017)	- / 11.3	274 mW/ m^3	-	1.68 %	90-95 %
Song et al. (2017)	12.4 / 35.3	150mW/ m^3	560 mv (average)	0.31 %	94.90 %
Xu et al. (2017)	2 / 5	2000 mW/ m^3	590 mV (maximum)	1.85 %	88.70 %
Wang et al. (2017)	1.43 / 4.08	5.11 mW/ m^2	183.2 mV (average)	-	52.29 %

3.3.3 CW-MFCs for COD removal

Apart from the generation of electricity, CW-MFCs are linked to high organic matter removal efficiencies. As it is summarized in Table 3.4 COD removal efficiencies reported ranged from 44.5±3.3% (Wang et al., 2016) and 100% (Oon et al., 2015). However, these results are not comparable to real-scale HSSF-CWs' COD removal efficiencies which are generally lower (between 66 and 89 % of COD removal is reported for real-scale SSF CWs in Puigagut et al., 2007b). This is due to the fact that CW-MFCs do not reproduce HSSF-CWs redox conditions, especially those CW-MFCs that impose high redox conditions at the cathode by means of external aeration. As it has been previously discussed, upflow designs force wastewater to flow from the anode to the cathode in which, if aerated, organic matter is completely removed by means of aerobic oxidation pathways leading to removal efficiencies up to 100%.

Furthermore, the implementation of MFCs in CWs can also lead to an improvement of CWs treatment efficiencies which constitutes one of its more attractive environmental applications. This is due to the fact that the increasing of CWs' treatment rates could lead to the reduction of their surface thus alleviating one of their major drawbacks. The improvement of the treatment rate as a consequence of the implementation of MFCs and the generation of an electrical circuit within the system is possible because the anode, which is connected to the cathode, constitutes an electron acceptor with a high redox potential in a very anaerobic environment (Srivastava et al., 2015). Therefore, there is an energy gain for bacteria when using the anode to transfer the electrons that result from the oxidation of the organic compounds. Anaerobic degradation pathways are the predominant organic matter removal processes in CWs. They are described to be slower and less efficient than the aerobic oxidation. This is due to the scarcity of electron acceptors in anaerobic areas of CWs. In this context, the anode constitutes not only a high redox potential but also a non-consumable electron acceptor in such competitive environment. Therefore, it can contribute in organic matter treatment improvement (Huang et al., 2011; Srivastava et al., 2015). Also the presence of an external electrical circuit stimulates the establishment of exoelectrogenic communities on anodic biofilms (Larrosa-Guerrero et al., 2010).

Accordingly, MFCs fostered the degradation of the organic matter leading to better wastewater quality at the effluents of the connected conventional MFCs when compared to the unconnected ones (Katuri et al., 2011). The same results were found for CW-MFCs when operated with glucose in which closed circuit systems showed 16-20% higher COD removal efficiencies when compared to open circuit CW-MFCs (Srivastava et al., 2015). Furthermore, Srivastava et al. (2015) assessed differences between connected systems and normal-CWs and reported 10-31% higher performances. Smaller differences were found by Oon et al. (2016) in CW-MFCs operated with synthetic wastewater, who reported 8% higher removal efficiencies when the system was operated under closed-circuit. The enhancement of anaerobic degradation as a consequence of MFCs implementation has also been studied for sediment and conventional MFCs. In this context, Huang et al. (2011) and Larrosa-Guerrero et al. (2010) reported for soluble COD, 7.1 times and 22% higher removal rates in closed-circuit conditions, respectively.

3.3.4 CW-MFCs for bioindication purposes

Microbial Fuel Cells are bioelectrochemical systems that generate electricity from the oxidation of organic compounds by means of exoelectrogenic bacteria as catalysts (Logan et al., 2006). Conventional MFCs have been described as a suitable technology to become an online tool for organic matter concentration assessment. This is because MFCs directly provide an electrical signal that can be correlated to organic wastewater content (Peixoto et al., 2011). The main advantages resulting from the utilization of MFCs as biosensor devices are the possibility of in-situ implementation, the on-line monitoring and the avoiding of complex laboratory procedures requiring chemicals addition and the lack of transducer necessity (Di Lorenzo et al., 2009; Peixoto et al., 2011). So far several authors have already proved the viability of using this technology for biosensing purposes both for synthetic and real wastewater COD assessment (Chang et al., 2004; Di Lorenzo et al., 2009).

Linear relationships between influent organic matter concentration and the electrical output obtained with Rsquare values over 0.9 are reported (Di Lorenzo et al., 2009; Gonzalez del Campo et al., 2013; Kim et al., 2003a;

Peixoto et al., 2011). More into detail, Di Lorenzo et al. (2009), using a conventional air-cathode MFCs, found linear relationships between COD content of both real and synthetic wastewater (in the range of 40 and 200 mgO_2/L) and current generated. Apart from the relationship that can be stablished between the electric response of the system and the organic matter concentration in the medium tested, there are other parameters which are of importance when bioindicating. Accordingly, response time is a crucial parameter for biosensing tools, especially for real-time monitoring (Liu and Mattiasson, 2002). In literature there are reported response times of 60 min when using conventional MFCs fed with glucose and glutamic acid as MFCs carbon source (Chang et al., 2004). Much lower response times (between 3 and 5 minutes) were achieved also using glucose as the carbon source (Kumlanghan et al., 2007). However, wastewater is composed by complex carbohydrates that need to be hydrolyzed to volatile fatty acids before being utilized by exoelectrogenic bacteria (Kiely et al., 2011) and therefore, the response time of systems fed with wastewater is significantly higher. Systems operated with wastewater were linked to response times of about 10h for concentrations higher than 78±8 mgO2/L (Peixoto et al., 2011).

If we focus on CW-MFCs some authors observed an increase in the electric output when higher organic loads were supplied to the cells (Oon et al., 2016; Srivastava et al., 2015). Also the result of increasing organic matter concentrations were tested with CW-MFCs (Liu et al., 2014). Increasing power densities were recorded when influent COD concentration where in the ranged between 50 and 250 mg O_2/L. However, when influent concentration went from 250 to 1000 mg O_2/L, due to the intrusion of organic matter in the cathodic chamber, the electric output decreased (Liu et al., 2014). Wang et al. (2017) also found an increasing relationship between inlet COD concentrations and average voltages generated. Although positive results in CW-MFCs were obtained, none of this research was conducted with bioindication purposes.

3.3.5 Life Cycle assessment

LCA is a standardized methodology for the evaluation of the potential environmental impacts generated by a product, process or service using a cradle to grave approach (ISO, 2000; ISO, 2006). It identifies and quantifies

the environmental burdens associated with energy and materials used (inputs) and waste released into the environment (outputs) during the whole life cycle. LCA is mainly used to compare different competing products or technologies, as well as to identify improvement alternatives for a single product or technology.

CW-MFCs provide an energy surplus that can partially cover the energy input necessary for wastewater treatment. Moreover, they can stimulate the degradation of organic matter present in wastewater by fostering more efficient degradation pathways carried out by exoelectrogenic bacteria (Katuri et al., 2011; Srivastava et al., 2015). As a consequence, the implementation of MFCs in HSSF CWs could improve CWs treatment efficiency and reduce their surface requirement. However, materials used for conventional MFCs electrodes (e.g. carbon fiber, membranes, stainless steel) are expensive materials with poor environmental performance (Foley, et al., 2010; Gude, 2016; Liu and Cheng, 2014; Zhou et al., 2011). Therefore, although energy inputs and surface area requirement may be reduced, both costs and environmental impacts could significantly increase when implementing MFCs in CW treatment systems.

Several studies which analyze the environmental impacts of CW systems have been carried out (Dixon et al., 2003; Fuchs, et al., 2011; Machado et al., 2007; Yildirim et al., 2012). Also a LCA has been conducted to compare MFCs to different wastewater treatment options (Foley et al., 2010). Accordingly, positive environmental impacts reported of using MFCs as wastewater treatment units arose from the generation of electricity. However, resource and emission intensive materials are outlined to be required for MFCs construction leading to high environmental impacts. Conclusions taken from the LCA conducted to a plant MFC (P-MFC) were the same. P-MFC showed a worse environmental performance than conventional electricity production technologies due to limited electrical output of P-MFC combined with the large impact materials used. However, there is still no study assessing environmental impacts of CW systems coupled with MFCs.

CW-MFC architecture and operational conditions

This chapter is based on the following articles:

Corbella, C., Garfí, M., & Puigagut, J. (2014). Vertical redox profiles in treatment wetlands as function of hydraulic regime and macrophytes presence: Surveying the optimal scenario for microbial fuel cell implementation. *Science of the Total Environment*, *470–471*, 754–758.

Corbella, C., Guivernau, M., Viñas, M., & Puigagut, J. (2015a). Operational, design and microbial aspects related to power production with microbial fuel cells implemented in constructed wetlands. *Water Research*, *84*, 232–242.

Corbella, C., Garfí, M., & Puigagut, J. (2016b). Long-term assessment of best cathode position to maximise microbial fuel cell performance in horizontal subsurface flow constructed wetlands. *Science of The Total Environment*, *563–564*, 448–455.

Corbella, C., Steidl, R. P., Puigagut, J., & Reguera, G. (2017). Electrochemical characterization of *Geobacter lovleyi* identifies limitations of microbial fuel cell performance in constructed wetlands. *International Microbiology*, *20*(2), 55–64.

In this chapter all the investigations concerning CW-MFCs architecture and operational conditions are gathered. The chapter is divided in four different sections corresponding to the four different publications referenced in the previous page. As a consequence, the reader will find the formal structure of an article four consecutive times in this chapter.

Within this articles redox profiles, hydraulic regime, presence of plants, primary treatment applied, evapotranspiration effect in horizontal subsurface constructed wetlands are investigated and discussed in order to maximize the benefits from MFCs implementation. In terms of CW-MFCs architecture the optimal distance between electrodes, the anode to cathode surface ratio and optimal cathode position are assessed. All the architecture and operational parameters here described were studied by means of the pilot plant CW-MFCs.

The influence of architecture and operational conditions on microbial biofilms was also studied. Therefore, this chapter also includes all the experiments conducted in order to deeply understand microbiological interactions within CW-MFCs biofilms. This experiments were published in three different articles (4.1, 4.2 and 4.3). In article 4.2 anodic biofilms from the pilot-plant CW-MFCs were analyzed and a predominant exoelectrogen was detected. A deep insight on the characterization of this exoelectrogen was conducted by means of conventional MFCs and the conclusions are also introduced here (article 4.4).

4.1 Vertical redox profiles as function of hydraulic regime and macrophytes presence

Abstract

Sediment microbial fuel cell (sMFC) represents a variation of the typical configuration of a MFC in which energy can be harvested via naturally occurring electropotential differences. Moreover, constructed wetlands show marked redox gradients along the depth which could be exploited for energy production via sMFC. In spite of the potential application of sMFC to constructed wetlands, there is almost no published work on the topic. The main objective of the present work was to define the best operational and design conditions of sub-surface flow constructed wetlands (SSF CWs) under which energy production with microbial fuel cells (MFC) would be maximized. To this aim, a pilot plant based on SSF CWs treating domestic sewage was operated during six months. Redox gradients along the depth of SSF CWs were determined as function of hydraulic regime (continuous vs discontinuous) and the presence of macrophytes in two sampling campaigns (after three and six months of plant operation). Redox potential (E_H) within the wetlands was analysed at 5, 15 and 25 cm. Results obtained indicated that the maximum redox gradient was between the surface and the bottom of the bed for continuous planted wetlands (407.7±73.8 mV) and, to a lesser extent, between the surface and the middle part of the wetland (356.5±76.7 mV). Finally, the maximum redox gradients obtained for planted wetlands operated under continuous flow regime would lead to a power production of about 16·mW/m².

4.1.1 Introduction

Sub-surface flow constructed wetlands (SSF CWs) are used for the wastewater treatment in small communities worldwide (García et al., 2010; Puigagut et al., 2007b). Low cost operation and maintenance, low energy requirements and good landscape integration are some of the most attractive advantages of this technology compared to conventional treatment systems (García et al., 2003; Rousseau et al., 2008). Contaminant removal efficiency in SSF CWs depends, among other factors, on redox potential (E_H) conditions (Caselles-Osorio and García, 2007; Pedescoll et al., 2011b). Accordingly, E_H in SSF CWs is subjected to great spatial and temporal variation, which is caused, in turn, by several factors including the presence of plants, fluctuations in the water level due to evapotranspiration, light intensity and temperature (Białowiec et al., 2012; Dušek et al., 2008; García et al., 2010; Wiessner et al., 2005). In general terms, E_H variation in SSF CWs is accepted to be of great intensity (fluctuation of several hundreds of millivolts within few hours) (Dušek et al., 2008) in depth rather than across the length of the bed (García et al., 2003), and especially pronounced within the morning hours (Białowiec et al., 2012). SSF CWs can be designed, by changing operational and design parameters, to favour a desired range of redox conditions targeting a specific pollutant removal pathway (Faulwetter et al., 2009) or, more recently, to produce energy through microbial fuel cell (MFC) implementation (Yadav et al., 2012).

To this regard, in an MFC bacteria oxidize an electron donor (organic matter), with an anodic electrode as the electron acceptor. The electrons flow from the anode through an electrical circuit toward a high redox electron acceptor, such as oxygen, at the cathode. Furthermore, a sediment microbial fuel cell (sMFC) represents a variation of the typical configuration of a MFC in which energy can be harvested via naturally occurring electropotential differences (Reimers et al., 2001; Whitfieldl, 1972). So far, sMFC have been successfully implemented in rice paddy fields (Kaku et al., 2008) and marine environments (Bond et al., 2002; Holmes et al., 2004; Lowy et al., 2006; Reimers et al., 2001; Rezaei et al., 2007; Ryckelynck et al., 2005; Tender et al., 2002; Whitfieldl, 1972), and just one work has been published on the implementation of sMFC to constructed wetlands (though it is a laboratory study using dye wastewater and operated in batch mode) (Yadav et al., 2012).

To the best of our knowledge, there are no current studies addressing the influence of operational and design parameters in SSF CW (such as hydraulic regime and macrophytes presence) on redox profiles with the aim at describing the best scenario for sMFC implementation. Therefore, the potential use of SSF CW for energy production remains unaddressed in current literature. The hypothesis behind this work is whether constructed wetlands are a suitable technology for energy production via microbial fuel cell implementation.

The objective of the present work was to determine the influence of flow regime and macrophytes presence on redox conditions along the depth of SSF CWs. The analysis of redox gradients along the depth of SSF CWs will be used to describe the optimal scenario to maximize the energy production with sMFC.

4.1.2 Materials and Methods

4.1.2.1 Pilot plant

The experimental plant was located in Barcelona, and treated urban wastewater directly pumped from a municipal sewer. It was built in 2011 at the Department of Hydraulic, Maritime and Environmental Engineering of the Universitat Politècnica de Catalunya and was set in operation in March 2011.

The wastewater was coarsely screened and subsequently stored in a 1.2 m^3 plastic tank, which was continuously stirred in order to avoid sedimentation of solids. This tank was equipped with level buoys that controlled the operation of the feeding pumps. Wastewater retention time in this tank was approximately 5 h. From the storage tank, the wastewater was pumped to 4 cylindrical PVC static settlers. Two settlers of 14 L fed the wetlands working under continuous flow regime, while the other two settlers (of 7 L each) fed the wetlands working under discontinuous flow regime.

From settlers, wastewater was conveyed to the secondary treatment which consisted of six SSF CWs of 0.4 m^2 each, fed with 21 L day^{-1} of primary settled domestic sewage. Wetlands were constructed in plastic containers 0.75 m long, 0.55 m wide and 0.39 m high. A uniform gravel layer (40% initial

porosity) was used which provided a wetland depth of 0.35 m. The water level was kept 0.05 m below the gravel surface to give a water depth of 0.30 m.

Each experimental condition was tested in duplicate. All the wetlands were operated at 2.5 days of hydraulic retention time (HRT) and at an organic loading rate (OLR) of about 5 gBOD. m^{-2} day^{-1}. Four of the six wetlands were fed under continuous flow regime (cf SSF CW) and the two wetlands remaining were fed discontinuously (3 times per day) (df SSF CW). cf SSF CW received a flow of 0.875 L h^{-1}, while df SSF CW were fed three times a day with a flow of 7 L per pulse by means of electrovalves. Furthermore, two of the cf SSF CW and the df SSF CW wetlands were planted with common reed *(Phragmites australis)* in March 2011 at an initial density of 16 plants m^{-2}. Effluent was discharged into a graduated tank of 22 L capacity. Measuring the influent and effluent flow allowed us to calculate wetlands efficiency on a mass balance basis. Overall, the influence of the presence of macrophytes on redox conditions within the wetlands was addressed by comparing the planted and unplanted wetlands operated under continuous flow regime, whereas the effect of flow regime was addressed by comparing the planted wetlands operated under continuous or discontinuous flow regime.

4.1.2.2 *Redox potential monitoring*

Redox conditions within the wetlands were monitored in two sampling campaigns during sampling periods of 24 hours for each wetland. The first sampling campaign was conducted in June 2011 (after three months of plant operation) and the second campaign was conducted in September 2011 (after six months of plant operation). It is worth mentioning that the root system was approximately 20 cm deep during the first sampling campaign whereas it reached the bottom of the wetland (approximately 30 cm) during the last sampling campaign. Vertical redox profiles were obtained by means of three redox probes (Digimed TH-404) equipped with a platinum electrode (Ag/AgCl reference system - accuracy: ± 10 mV). Redox probes were inserted at each wetland at 5, 15 and 25 cm depth and connected to a data logger (DATA TAKER DT50 series 3) that recorded redox values every ten seconds for periods of 24 hours. Redox data was transformed to express the results in terms of the standard hydrogen electrode (E_H).

4.1.2.3   Physical and chemical analyses

The systems were analysed from March to October 2011 for their efficiency on contaminant removal. Surveyed water quality parameters were soluble COD, total COD, ammonia nitrogen and nitrate nitrogen. Analyses were carried out once a week according to Standard Methods (APHA, 2005).

4.1.2.4   Power production estimation

According to Logan et al. (2006), the electromotive force of the MFC (in volts), which is actually the maximum attainable voltage, can be expressed as (equation 1):

$$E_{emf} = E_{cat} - E_{an}$$

According to Logan et al. (2006), the measured cell voltage (E_{cell}) is usually lower than the electromotive force of the cell (E_{emf}) due to a number of losses such as anode and cathode overpotentials (η_a and η_c, respectively) and ohmic losses (IR_Ω) (equation 2):

$$E_{cell} = E_{emf} - \left(\sum \eta_a + \left|\sum \eta_c\right| + IR_\Omega\right)$$

For the purposes of this section the cell voltage (E_{cell}) will be considered the 50% of the electromotive force of the MFC (E_{emf}) (Logan et al., 2006). Although it is just a rough estimation of the actual cell voltage that could be obtained in SSF CWs, authors believe that it is a good approximation to address which is the potential power production of MFC implemented in SSF CWs.

Furthermore, according to Logan et al. (2006), the theoretical power produced by the implementation of a microbial fuel cell is that of (equation 3):

$$P = I \cdot E_{cell}$$

Normally the voltage is measured across a fixed external resistor (R_{ext}), while the current is calculated from Ohm's law (equation 4):

$$I = \frac{E_{cell}}{R_{ext}}$$

Thus, power is usually calculated as (equation 5):

$$P = \frac{E_{cell}^2}{R_{ext}}$$

Power (P) was finally normalized to the projected surface of an anode (A_{an}) (Cheng et al., 2006; Rezaei et al., 2007), which in our case could be also considered as wetland area (equation 6).

$$P = \frac{\frac{E_{cell}^2}{R_{ext}}}{A_{an}}$$

4.1.3 Results and discussion

4.1.3.1 Wastewater quality

Quality parameters at the influent of the treatment plant were that of 0.72±0.03, 5.7±0.8, 9.5±0.07 and 0.2±0.13 g· m^{-2} d^{-1} to ammonia, soluble COD, total COD and nitrates, respectively. Ammonia removal efficiencies were higher for planted systems (ca. 85%) when compared to unplanted systems (ca. 40%). More precisely, planted SSF CWs operated under continuous flow regime showed an average effluent load of 0.1±0.05 gm^{-2}d^{-1}, whereas the unplanted system provided effluents at an average effluent load of 0.46±0.08 g· m^{-2} d^{-1}. These results are in accordance with previous studies, which demonstrated that nitrogen removal was enhanced in planted systems, either directly through assimilation or indirectly through the influence of plants on oxygen, microbial activity and nitrification (Brisson and Chazarenc, 2009).

Average effluent load ranged from 1.75 to 2.9 g· m^{-2} d^{-1}, from 1.4 to 1.54 g· m^{-2} d^{-1} and from 0.08 to 0.1 g· m^{-2} d^{-1} for total and soluble COD and nitrates, respectively, without significant differences among treatment conditions. It is necessary to point out that nitrates removal was generally low, regardless the experimental conditions considered (about 50% removal). Low nitrate removal for the surveyed systems is probably due to the higher redox conditions described in shallow wetlands (García et al., 2005).

4.1.3.2 *Redox vertical distribution within the wetlands as function of operation and design conditions*

Influence of hydraulic regime on redox conditions

The influence of hydraulic regime was addressed by contrasting the results obtained from the redox profiles of the planted wetlands operated under continuous or discontinuous hydraulic regime. It is generally accepted that hydraulic regime influences to a great extent the redox status in constructed wetlands (Faulwetter et al., 2009). Accordingly, it was demonstrated that intermittent flow regime creates a temporal E_H variation and imposes higher E_H conditions when compared to continuous flow regime (Allen et al., 2002; Caselles-Osorio and García, 2007). Our results suggest that the redox conditions at the surface of the wetland were unaffected by the sort of hydraulic regime applied, presenting redox values of ca. +200 mV (Figure 4.1.1). However, a wetland operated under discontinuous flow regime presented higher redox conditions either at the middle and bottom sections of the wetland when compared to the system operated under continuous flow (Figure 4.1.1). More precisely, redox conditions at the bottom of the wetlands were about 125 mV higher for discontinuous flow when compared to continuous flow (Figure 4.1.1). Furthermore, discontinuous flow wetlands not only showed higher daily average redox conditions at middle depths when compared to continuous flow wetlands but also showed higher daily variation of redox (Figure 4.1.2). To this regard, redox decrease in discontinuous flow wetlands showed a marked drop in redox conditions (between 50 and 200 mV drop) that coincided with feeding episodes (arrows marked in Figure 4.1.2).
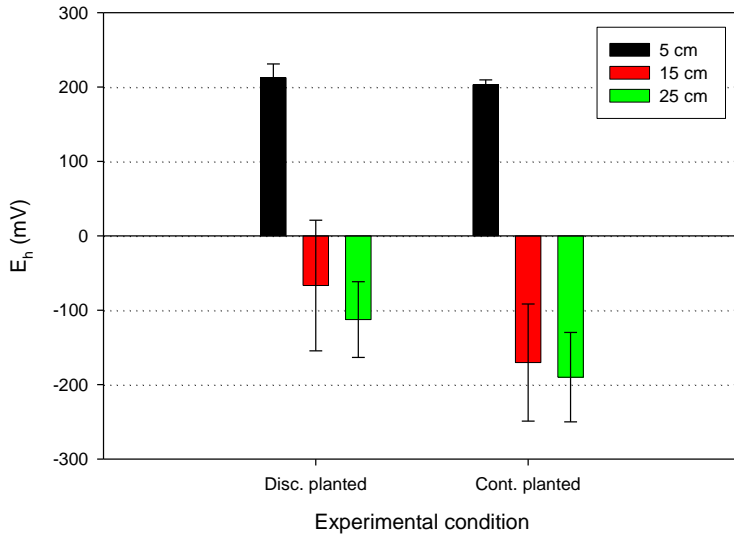
Figure 4.1.1 Effect of flow regime. Daily average redox potential recorded at 5 cm, 15 cm and 25 cm depth in planted wetlands operated under continous and discontinuous flow. Note that bars are depicted by using the data obtained from wetlands replicates during both experimental sampling campaigns (for each bar n=96).
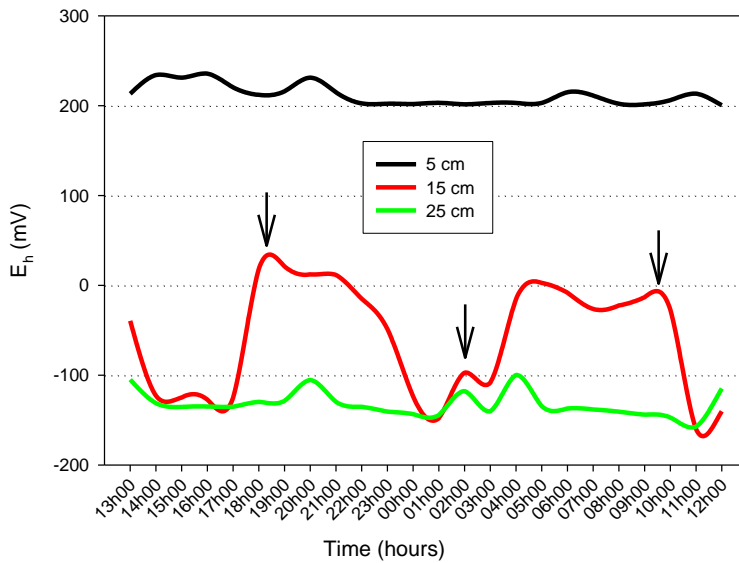
Figure 4.1.2 Daily redox variation of the wetland operated under discontinuous flow regime in planted systems. Note: arrows represent feeding episodes.

Influence of vegetation on redox conditions

The influence of vegetation was addressed by comparing the results obtained from the planted vs unplanted wetlands operated under continuous flow regime during the last sampling campaign (where plants root system reached the bottom of the wetland). Accordingly, it is widely accepted that the redox status in constructed wetlands is affected by both the presence and species of the plants (Białowiec et al., 2012; Dušek et al., 2008; Gagnon et al., 2007). Overall, the presence of plants in constructed wetlands stimulates higher E_H conditions when compared to unplanted wetlands (Tanner, 2001), although the influence of the rhizosphere on redox conditions is that of just few millimetres away from the roots (Bezbaruah and Zhang, 2005). In our study, redox conditions were clearly unaffected by the presence of plants at 5 cm and at 25 cm depth (Figure 4.1.3). However, at 15 cm depth the redox was lower (between 100 and 125 mV lower) for the planted wetlands than for the unplanted. Authors believe that, since planted wetlands have been described to enhance both bacterial density and biomass (Collins et al., 2004; Gagnon et al., 2007), the redox conditions in the bulk liquid (which is actually the place where we measured the redox) could be lower as a result of a higher bacterial activity. It is worth mentioning that the effect of the presence of macrophytes here considered might be underestimated to some extent due to the immaturity of the systems (six-months-old wetlands). Accordingly, longer study periods are required to fully determine the influence of the presence of macrophytes on the redox distribution along the depth of wetlands.
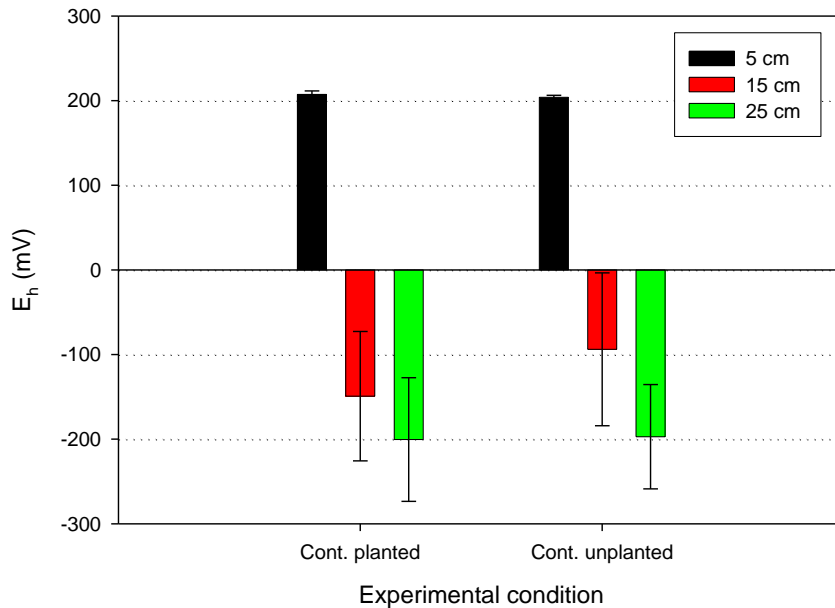
Figure 4.1.3 Effect of macrophytes. Daily average redox potential recorded at 5 cm, 15 cm and 25 cm depth for planted and unplanted wetlands operated under continous flow. Note that bars are depicted by using the data obtained from wetlands replicates during the last sampling campaign (for each bar n= 48).

<u>Redox gradient along the depth of the bed</u>

In general terms, redox conditions on the surface of the wetland (5 cm depth) and at the bottom part of the wetland (25 cm depth) varied to a lesser extent along the day than at middle depths (15 cm depth) which is in accordance to that described elsewhere (Dušek et al., 2008). More precisely, at 15 cm depth daily fluctuation of E_H ranged between -300 and +50 mV and was especially pronounced in planted wetlands working under discontinuous flow regime (Figure 4.1.2). The daily average redox gradient along the depth of the wetland was maximal between the surface of the wetland and the bottom and, to a lesser extent, between the surface and the middle depth, regardless the operational or design conditions applied (Table 4.1.1). Finally, the redox gradient between the middle depth and the bottom was the minimal regardless the experimental conditions tested (Table 4.1.1). Overall, the operational condition that provided the greatest redox gradient was that of the planted wetlands operated under continuous flow regime (Table 4.1.1).

Table 4.1.1 Average daily values of E_H gradient and power density calculated for all CWs according to their operational conditions. [a] Power is calculated assuming a 50% efficiency (difference between E_{cell} and E_{emf}) and using equation 6 in the text (external resistance of the circuit and the surface of the anode is that of 1000 ohms and 0.0041 m², respectively (Yadav et al., 2012). [b] Related to both anode and wetland surface.

Operational conditions		E_H gradient[a] (mV)			[a]Power (mW/m^{2}[b])		
		5-15 cm	5-25 cm	15-25 cm	5-15 cm	5-25 cm	15-25 cm
Continuous flow	Planted	356.5±76.7	407.7±73.8	51.2±53.4	14.5±7.7	16.1±6.7	0.7±1.1
	Unplanted	297.7±90.5	401±61	103.3±32.9	6.9±4.5	13.7±6.8	1.4±0.7
Discontinuous flow	Planted	255.2±64.1	326.2±49.3	71±37.8	8.3±3.4	10.4±3.3	0.8±0.6

4.1.3.3 Theoretical power production of microbial fuel cells implemented in constructed wetlands

In this study vertical redox profiles in SSF CWs were determined to estimate the optimal configuration under which the energy production with MFCs would be maximized (maximum redox gradient).

The redox gradients (E_{emf}) calculated are summarized in Table 4.1.1. Moreover, assuming that the external resistance of the circuit would be close to 1000Ω (Yadav et al., 2012) and that the electrodes of the MFC would occupy a surface of 41 cm^2 of the wetland (Yadav et al., 2012), the estimated power production according to each of the considered redox gradients is also shown in Table 4.1.1. Note that we assume that E_{cell} will be reduced by a 50% from the E_{emf} due to potential losses (Logan et al., 2006). Accordingly, the maximum theoretical power production by means of a MFC implemented in a constructed wetland would be that of 16.1 mW/m^2 (Table 4.1.1). From the results shown in Table 4.1.1 it is possible to conclude that the redox gradient would be always maximal (and so the power production) between the surface of the wetland and the bottom (between 50 to 100 mV higher than the redox gradient between the surface and the middle depth); resulting in the planted wetlands operated under continuous flow being the best configuration to maximize power production with MFC. Moreover, assuming that the required surface area per person equivalent in SSF CWs is that of 5 m^2 (Kadlec and Wallace, 2009), the power production in a MFC implemented in a planted SSF CW operated under continuous flow regime would be up to 80.5 mW/PE.

4.1.4 Conclusions

Ammonia removal was higher (ca. 85%) for planted wetlands than for unplanted wetlands (ca. 40%), regardless the flow regime considered. Furthermore, nitrates and organic matter removal was not significantly different for any wetland configuration here considered.

Redox conditions at the surface of the wetland were unaffected by the hydraulic regime applied. However, redox conditions either at the bottom or at middle sections of the wetland were higher for discontinuous flow when compared to continuous flow.

Planted wetlands showed lower redox conditions (between 50 to 100 mV lower) at 15 cm depth than those left unplanted, whereas redox conditions at 5 cm and 25 cm depth were not significantly different between planted and unplanted wetlands.

The daily average redox gradient along the depth of the wetland was maximal between the surface of the wetland and the bottom and, to a lesser extent, between the surface and the middle depth, regardless the operational or design conditions applied. Overall, the operational conditions that provided the greatest redox gradient were between the surface and the bottom of planted wetlands operated under continuous flow regime (407.7±73.8 mV of gradient).

Theoretical power production by means of a MFC implemented in a constructed wetland was estimated to be that of 16.1 mW/m^2, which would be equivalent (under certain assumptions) to 80.5 mW/PE.

4.2 Operational, design and microbial aspects related to power production with microbial fuel cells implemented in constructed wetlands

Abstract

This work aimed at determining the amount of energy that can be harvested by implementing microbial fuel cells (MFC) in horizontal subsurface constructed wetlands (HSSF CWs) during the treatment of real domestic wastewater. To this aim, MFC were implemented in a pilot plant based on two HSSF CW, one fed with primary settled wastewater (Settler line) and the other fed with the effluent of a hydrolytic up-flow sludge blanket reactor (HUSB line). The eubacterial and archaeal community on wetland gravel, MFC electrodes and primary treated wastewater was profiled by 16S rRNA gene-based 454-pyrosequencing and qPCR of *16S rRNA* and *mcrA* genes. Maximum current (219 mA/m^2) and power (36 mW/m^2) densities were obtained for the HUSB line. Power production pattern correlated well with water level fluctuations within the wetlands, whereas the type of primary treatment implemented had a significant impact on the diversity and relative abundance of eubacteria communities colonizing MFC. It is worth noticing the high predominance (13-16% of relative abundance) of one OTU belonging to *Geobacter* on active MFC of the HUSB line that was absent for the settler line MFC. Hence, MFC- show promise for power production in constructed wetlands receiving the effluent of a HUSB reactor.

4.2.1 Introduction

Microbial Fuel Cells (MFC) are bioelectrochemical systems that generate current by means of electrochemically active microorganisms as

catalysts. In a MFC, organic and inorganic substrates are oxidized by bacteria and the electrons are transferred to the anode from where they flow through a conductive material and a resistor to a higher redox electron acceptor, such as oxygen, at the cathode (Logan et al., 2006; Rabaey et al., 2007). Different extracellular electron transfer (EET) mechanisms proposed can be divided in two main mechanisms; a) direct electron transfer (DET) and b) indirect electron transfer (IET). DET is based on the physical contact between the microbial outer membrane (OM) proteins, such as c-type cytochromes (Holmes et al., 2006; Reguera et al., 2005), or a conductive nanowires or pili (Gorby et al., 2009; Reguera et al., 2005), and the anode electrode surface. In IET, direct contact between the microbes and the electrode surface is not required, and in this case soluble electron shuttles or electron mediator compounds are involved in this process. A range of electron mediators produced by bacteria have been reported, such as melanin, phenazines (Rabaey et al., 2005), flavins and quinones (Freguia et al., 2009). So far, there are two well-known bacterial genera which present exoelectrogenic activity in pure culture, i.e., *Shewanella* (Ringeisen et al., 2006) and *Geobacter* (Kiely et al., 2011; Richter et al., 2008). To date, a high diversity of microorganisms has been described to perform anode respiration to a certain degree (Logan, 2009). Over 20 different exoelectrogenic bacteria have been reported in the last decade, belonging to diverse phylogenetic groups: alpha-proteobacteria (*Rhodopseudomonas, Ochrobactrum* and *Acidiphilium*), beta-proteobacteria (*Rhodoferax, Comamonas*), gamma-proteobacteria (*Shewanella, Pseudomonas, Klebsiella, Enterobacter and Aeromonas, Citrobacter*), delta-proteobacteria (*Geobacter, Geopsychrobacter, Desulfuromonas* and *Desulfobulbus*), Epsilon-proteobacteria (Arcobacter), Firmicutes (*Clostridium* and *Thermincola*), Acidobacteria (*Geothrix*) and Actinobacteria (*Propionibacterium*) (Logan, 2009; Xing et al., 2010). However, the power density achieved in most of the experiments working with mixed cultures is higher than in pure cultures (Nevin et al., 2008; Rabaey et al., 2004; Rabaey and Verstraete, 2005). These results reinforce the idea that increased electricity generation could be attributed to synergistic interactions within the microbial community. Namely, there could be microorganisms that do not exchange directly electrons with the electrode, but could be settling up interactions among other members of the microbial community playing a crucial role not only in the operation of a MFC but also on its performance improvement (specially under the presence of complex organic substrates

such as wastewater) (Borole et al., 2011 and references therein). Methanogens such as *Methanosaeta* and *Methanosarcina* are, for example, routinely detected in mixed species, anode biofilms of MFC, where they presumably promote syntrophic interactions with exoelectrogenic eubacteria in the anode biofilm (Chung and Okabe, 2009; Rotaru et al., 2014a, 2014b; Sotres et al., 2015).

Compounds oxidized at the anode are mainly simple carbohydrates such as glucose or acetate that can be already present in the environment or obtained from the microbial degradation of complex organic substrates such as organic sediments or wastewater (Min and Logan, 2004; Rabaey and Verstraete, 2005; Reimers et al., 2001). MFC are, therefore, an alternative technology to harvest energy directly from wastewater in the form of electricity (Du et al., 2007). In order to ensure the use of the anode as the final electron acceptor by electrochemical active microorganisms, no acceptor with higher redox potential shall be present in their vicinity (Lefebvre et al., 2011). Consequently, the electromotive force of the cell will depend on the redox gradient between the anode and the cathode (Logan et al., 2006; Rabaey and Verstraete, 2005).

To generate the redox gradient between electrodes, MFC require two separated areas that contain the anode (anaerobic area) and the cathode (aerobic area). In some aquatic environments there is the existence of natural redox gradients that can be exploited to produce energy via MFC implementation. So far, MFC have been mostly implemented in rice paddy fields (De Schamphelaire et al., 2008; Kaku et al., 2008) or marine sediments (Reimers et al., 2001; Rezaei et al., 2007). Furthermore, horizontal subsurface flow constructed wetlands (HSSF CWs) are engineered systems used for wastewater treatment that are subjected to great spatial redox variations (especially in depth) (García et al., 2003). Although the system is mainly anaerobic (Baptista et al., 2003), the very upper part of the wetland remains under aerobic conditions because its close contact with the atmosphere giving redox gradients of about 0.5 V vs SHE (Corbella et al., 2014; Dušek et al., 2008; García et al., 2003; Pedescoll et al., 2013a). As a result, natural redox gradients in HSSF CWs could be exploited to produce energy via MFC implementation, though only laboratory or small-scale based experiments with synthetic wastewater are currently available (Fang et al., 2013; Villaseñor et al., 2013; Yadav et al., 2012; Zhao et al., 2013). Furthermore, one of the

main problems of constructed wetlands is clogging (Pedescoll et al., 2011a). To prevent it, primary treatments are applied to wastewater. Generally, physical treatments such as settlers or Imhoff tanks are used. However, recently other technologies such as hydrolytic upflow sludge blanket (HUSB) reactors are being considered (Pedescoll et al., 2011b). A HUSB reactor prevents methane formation during organic matter hydrolysis due to a low HRT when compared to conventional anaerobic digesters (Ligero et al., 2001b). Moreover, HUSB reactors have the advantage over conventional settling of providing a higher concentration of biodegradable substrates (such as acetate) (Gonçalves et al., 1994) that can be easily removed in HSSF CWs. HUSB reactors as a primary treatment are of special interest in the context of MFC implemented in HSSF CW. Accordingly, HUSB reactors will provide a higher concentration of rapidly biodegradable substrate when compared to conventional settling, thus providing higher amount of fuel for MFC. This work aimed at determining the amount of energy that can be harvested by implementing MFC in HSSF CW during the treatment of real domestic wastewater. The effect of the type of primary treatment on power production, the daily and seasonal pattern of power production and the assessment of microbial populations associated to wastewater, electrodes (graphite) and CW materials (gravel) are also addressed.

4.2.2 Material and methods

4.2.2.1 Pilot plant

The pilot plant used in this study consisted of two horizontal subsurface flow constructed wetland. The wetlands were set up in March 2011 and had 0.4 m² of surface (70 cm length x 55 cm width). Wetlands were filled up with gravel (D_{60}=7.3; C_u=0.8) giving an initial porosity of 40%. Water level within the wetlands was kept at 30 cm (5 cm below the gravel surface). Both wetlands were planted with common reed (*Phragmites australis*), which were very mature at the moment this study was conducted (2.5 years after wetlands construction). Each wetland had a PVC cylinder of 20 cm diameter placed at the middle of the wetland that served not only to sample extraction but also to allocate the MFC.

The pilot plant was fed with urban wastewater pumped directly from the municipal sewer. Initially, wastewater was coarsely screened and after that it was pumped to a homogenisation tank of 1.2 m^3 where wastewater was continuously stirred in order to avoid solids sedimentation. After the homogenisation tank, wastewater was conveyed to the primary treatment. The primary treatment consisted of conventional settling for one of the wetlands and an anaerobic treatment based on a hydrolytic up-flow sludge blanket reactor (HUSB reactor) for the other. The HUSB reactor consisted of a PVC cylinder of 115 L of volume that was operated at 4 hours of HRT and at 10 g VSS/L. The settler consisted of two PVC cones of 14 L volume each that were operated in parallel. After the primary treatment, wastewater was pumped to the wetlands at a flow rate of 21 L/day, giving a design HRT of 2.6 days and an organic loading rate of 7.2 g.BOD$_5$.m^{-2}.day^{-1} and 6 g.BOD$_5$.m^{-2}.day^{-1} to the HUSB and Settler line, respectively.

4.2.2.2 *Microbial fuel cells*

Six MFC were set up for the purposes of the present work. Three of them were placed within the wetland fed by a HUSB reactor (HUSB_MFC) and the rest of them were placed within the wetland fed by the settler (SET_MFC) (Figure 4.2.1). Two of the three MFC for each wetland were in closed circuit whereas the other was left in open circuit and served as a control.

Each cell consisted of a cylinder of 40 cm in length and 5 cm in diameter made out of a plastic mesh filled with gravel up to a height of 35 cm (Figure 4.2.2). Both electrodes, anode and cathode, were placed within the cylinder at 15 cm and 5 cm below the water level, respectively. Thus, the distance between electrodes was 10 cm.

The anode and cathode were made out of 20 cylindrical graphite rods (1 cm length and 0.5 cm diameter each) wrapped with a stainless steel mesh marine grade 316L. Electrodes were 2.5 cm long, 3 cm wide and 1 cm high and squared shaped (Figure 4.2.2). The external circuit connected both electrodes by cooper wires and one external resistance of 1000 ohms. Epoxy resins were used to isolate connections from water.

Figure 4.2.1 Microbial fuel cells implemented within the wetland at the beginning of the experiment.
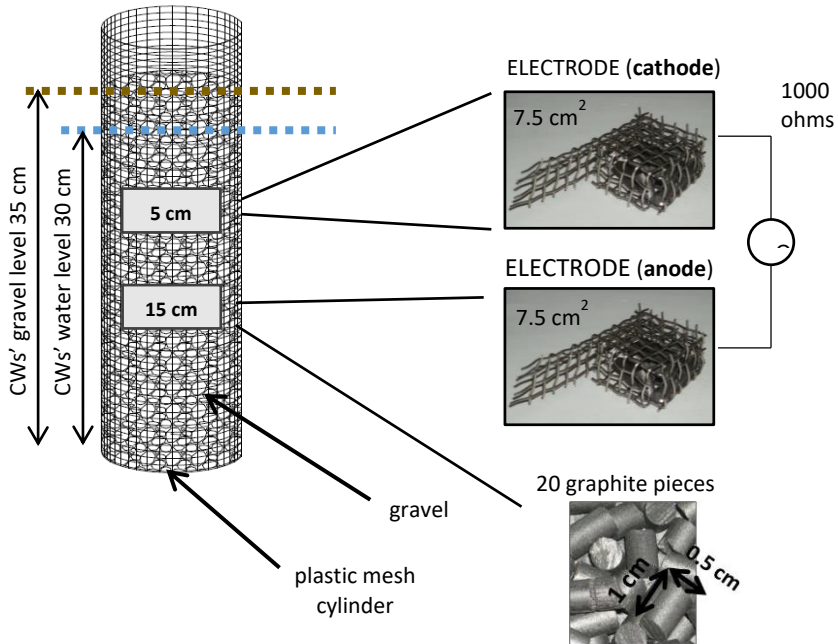
Figure 4.2.2 Outline of microbial fuel cells and electrodes

4.2.2.3 Redox, conductivity, temperature and water level measurements

Redox potential, water temperature, conductivity and water level were monitored all through the experiment. Redox potential was measured by means of an ORP probe (Ag/AgCl reference system - accuracy: ± 10 mV). Water level variation within the wetlands was determined using a pressure probe (TNS 119, Desin Instruments SA). Water temperature was measured using a Campbell Scientific 107-L Temperature Sensor. Finally, water conductivity was measured using a portable probe (Endress+Hauser CLM381). Water level variation and temperature were continuously measured while redox was alternatively measured in each wetland during periods of approximately 4 days. Both parameters were recorded by connecting the sensors to dataloggers (Datataker DT50 series 3) that stored one value every 15 minutes. Conductivity and pH were manually measured three times a week. Regarding redox potential measurement, two probes were placed just by the electrodes (at 5 cm (cathode) and 15 cm (anode) depth) and data obtained was transformed to express results in terms of the standard hydrogen electrode (E_H).

4.2.2.4 Voltage measurements

MFC were connected to a datalogger (Datataker DT50 series 3) which collected a value of voltage across the external resistance every 15 minutes. Voltage measurements were conducted in both lines from mid February to mid June 2013 (first period). After that, only the HUSB line was kept in operation until the end of the study period in July 2013 (second period).

4.2.2.5 Physical and chemical analyses

Water quality parameters surveyed during the experiment were BOD_5, total COD, soluble COD, ammonia, nitrate, nitrite, sulphate and orthophosphate. Analyses were performed according to Standard Methods (APHA, 2005). Sampling was conducted at the inlet, middle and outlet of the wetlands on a weekly basis. Water flow was also measured daily and removal efficiencies calculated on a mass balance basis.

4.2.2.6 Electrochemical characterization

Cell electromotive force was calculated according to Logan et al. (2006). Current was calculated following Ohm's law and power calculated according to:

$$P = V^2/R$$

Where,

V: is voltage across the resistance (in Volts)

R: external resistance (in Ohms)

All electrical data was related to the projected anodic area, which was considered to be the base of the electrode (7.5 cm^2) in order to express power production per wetland surface.

The maximum attainable voltage in a MFC (E_{emf}) is:

$$E_{emf} = E_{cat} - E_{an}$$

Where,

E_{emf}: electromotive force (in volts)

E_{cat}: is the redox at the cathode (in volts)

E_{an}: is the redox at the anode (in volts)

However, in a bioelectroquemical system cell performance is always affected by a number of losses that reduces the maximum attainable voltage (E_{emf}) to a cell voltage (E_{cell}) (Clauwaert et al., 2008; Logan et al., 2006). In the present work MFC efficiency was calculated as follows:

$$V_{eff} = \frac{E_{cell}}{E_{emf}} \cdot 100$$

It is important to note that average cell efficiency was calculated taking only into consideration E_{emf} values higher than 100 mV.

4.2.2.7 Microbial community assessment

DNA extraction

Wastewater samples from settler and HUSB were filtered in triplicate (5 mL each replicate) by means of Swinnex® Filter Holders (Millipore) with membrane filters of cellulose acetate (Whatman® 0.22 μm pore diameter). Filtrates were kept frozen at -20ºC until DNA extraction. Total DNA was extracted from influent wastewater filtrates, graphite material and gravel samples from both settler and HUSB lines. A bead beating DNA extraction was performed in triplicate by means of PowerSoil® DNA Isolation Kit (MoBio Laboratories, Solano Beach, CA, USA), following manufacturer's instructions.

Quantitative PCR assay

The ratios between eubacterial and methanogenic archaeal population were determined by quantifying the *16S* ribosomal *RNA* gene (*16S rRNA*) and the gene encoding of alpha subunit of methyl-coenzyme M reductase (*mcrA*). Gene copy numbers of *16S rRNA* and *mcrA* fragments were quantified in triplicate with the quantitative real time PCR (qPCR) as elsewhere described (Sotres et al., 2015). Standard curves were performed with known concentrations of the following reference cloned genes: *16S rRNA* gene from *Desulfovibrio vulgari*s subsp. *vulgaris* ATCC 29579, inserted in a TOPO TA vector (Invitrogen, Belgium); and a *mcrA* gene fragment obtained from *Methanosarcina barkeri* DSM 800 cloned a TOPO TA vector as well. The qPCR efficiencies of amplification were 92.2% and 90.4%, while the Pearson Correlation Coefficients (R^2) of the standard curves were between 0.999 and 0.971, and the slopes were between -3524 and -3575 for *16S rRNA* and *mcrA* gene, respectively.

All results were processed by MxPro™ QPCR Software (Stratagene, La Jolla, CA).

454-Pyrotag sequencing of total eubacterial and archaeal microbial populations

Massive bar-coded *16S rRNA* gene libraries targeting eubacterial region V1-V3 *16S rRNA* and archaeal region V3-V4 were sequenced utilizing

454 FLX Titanium equipment (Roche Diagnostics, Branford, CT, USA). In summary, diluted DNA extracts (1:10) were used as a template for PCR. Each DNA (two independent total DNA extract per sample) was amplified separately with both *16S rRNA* eubacteria and archaea set of primers containing unique multiplex identifier (MID) tags recommended by Roche Diagnostics (Roche Diagnostics, 2009). For eubacteria libraries the primer set were 27F (5'-AGRGTTTGATCMTGGCTCAG–3') and 519R (5'-GTNTTACNGCGGCKGCTG-3'), while archaeal set of primers were 349F (5'-GYGCASCAGKCGMGAAW-3') and 806R (5'–GGACTACVSGGGTATCTAAT-3'). The PCR conditions, subsequent purification and 454-pyrosequencing steps were performed as elsewhere described (Lladó et al., 2015). DNAs were sequenced utilizing Roche 454 FLX titanium instruments following manufacturer's guidelines.

Downstream 454-Pyrosequencing data analysis was carried out by using QIIME software version 1.8.0 (Caporaso et al., 2010) following a trimming protocol and grouping into Operational Taxonomic Units (OTUs) as previously described (Lladó et al., 2015). OTUs were taxonomically classified using BLASTn against GreenGenes database and compiled into each taxonomic level (DeSantis et al., 2006).

Data from pyrosequencing datasets was submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the study accession number SRP042796.

4.2.2.8 Assessment of cathode limiting conditions

Results from the main experiment suggested that MFC performance was limited by the cathode surface applied. In order to confirm this hypothesis a short experiment was conducted at the end of the study period. The experiment consisted of increasing the surface of cathode up to five times than that of the anode for two of the MFC implemented in the HUSB line. More precisely, the cathode to anode surface ratios tested were that of 1:1, 1:5, 1:4, 1:3, 1:2 and again 1:1. E_{cell} was recorded by means of a datalogger (Datataker DT50 series 3) which collected a value of voltage across the external resistance every 15 minutes. E_{cell} was measured for three days at each cathode to anode surface ratio tested.

4.2.2.9   Statistical analyses

Differences among experimental conditions for any of the considered parameters (physico-chemical parameters, redox conditions and cell voltage) were determined by carrying out ANOVA tests, T-tests and Wilcoxon tests depending on the type of dataset being compared. Data normality and homogeneity of variances were determined by performing the Kolmogorov-Smirnoff and Levenne tests, respectively. Differences among experimental conditions were considered significant at p values bellow 0.05. All statistical analyses were performed using the software package R 3.0.2, with the exception of statistical multivariate analyses (covariance- based Principal Component Analyses PCA) of pyrosequencing data which was analyzed by means XLSTAT 2014 software (Addinsoft, Paris, France).

4.2.3   Results and discussion

4.2.3.1   Treatment performance

No differences were found between experimental lines for ammonia, nitrate, nitrite, sulphate and orthophosphate. Ammonia removal was 60%, regardless the experimental line considered (Table 4.2.1). Ammonia removal efficiency in HSSF CW usually ranges from 40% to 55% (García et al., 2010). Higher ammonia removal rates here reported could be attributed to high evapotranspiration rates typical from small planted units (Pedescoll et al., 2013a; Tanner, 2001). Accordingly, water level variations impose higher redox conditions in wetlands which, in turn, may favour nitrification (García et al., 2010, 2003). In terms of organic matter, both concentrations of BOD and total COD were higher at the effluent of the HUSB reactor when compared to the settler; yet significant differences were only detected for the total COD. Hence, better removal rates for the settler line can be attributed to a lower organic loading rate when compared to the HUSB line. This result was already expected since the aim of the anaerobic reactor was to increase the total amount of biodegradable substrate supplied to the wetlands (Álvarez et al., 2008; Ligero et al., 2001). Moreover, samples extracted from the central part of the wetlands indicated that BOD_5, soluble and total COD in the vicinity of the MFC were significantly higher for the HUSB line when compared to the settler line. This result suggested that higher concentrations of substrate were

available for the HUSB_MFC which, in turn, could lead to a better performance of the cells (Cheng and Logan, 2011; Liu et al., 2004).

Table 4.2.1 Physical and chemical parameters measured. Note: average values are shown with standard deviation in brackets.

		HUSB LINE				SETTLER LINE			
		in	midd	out	rem (%)	in	midd	out	rem (%)
BOD$_{soluble}$ (n=7)	*(mg O$_2$ /L)*	137 (63)	71 (26)*	76 (63)	55	115 (53)	37 (14)*	42 (25)	54
COD$_{total}$ (n=17)	*(mg O$_2$ /L)*	323 (33)*	137 (53)*	126 (60)*	61	235 (19)*	100 (46)*	69 (29)*	71
AMMONIA (n=16)	*(mg NH4$^+$-N/L)*	41 (7)	-	19 (19)	58	39 (8)	-	17 (17)	60
NITRATE (n=13)	*(mg NO$_3$-N/ L)*	< 1	-	< 1	-	< 1	-	< 1	-
NITRITE (n=13)	*mg NO$_2$-N/ L*	< 1	-	< 1	-	< 1	-	< 1	-
SULPHATE (n=13)	*(mg SO$_4^{2-}$/L)*	102 (27)	-	68 (53)	34	113 (37)	-	68 (71)	40
ORTHOPHOSPHATE (n=13)	*(mg P-PO$_4^3$ /L)*	9 (3)	-	10 (7)	-	7 (2)	-	5 (5)	-

* Significant differences among treatment lines.

Furthermore, pH was mostly constant along the experiment and close to 7.5, regardless the type of primary treatment applied. Water temperature was, in average, 17.9 ± 5.2 ºC, with minimum values in February (6 ºC) and maximum values in July (28 ºC). Moreover, water temperature followed a daily cycle with temperature variations of about 2 ºC between day and night without significant differences among treatment lines.

Conductivity was significantly higher for the HUSB line when compared to the settler line. More precisely, conductivity was, in average, 2.69 ± 1.62 mS/cm and 3.37 ± 1.85 mS/cm in the settler and the HUSB line, respectively. Higher concentration of salts has been previously related to higher MFC performances (Cheng and Logan, 2011). This result suggested that cell performance could be higher for the HUSB line when compared to the settler line.

4.2.3.2 *Redox and voltage pattern*

Redox for both wetlands followed a very similar and conservative pattern. Redox potential at the anode was very constant and averaged -219 ± 29 mV (n= 1430) and -220 ± 46 mV (n=1177) for the HUSB and settler lines, respectively, without significant differences among treatment lines. Redox potential at the cathode described a very constant day-night pattern, especially pronounced for the HUSB line (Figure 4.2.3). Accordingly, during nightly hours the redox potential at 5 cm depth was almost as reduced as that of 15 cm depth and, therefore, the redox gradient was almost zero. However, during sunlight hours the redox potential increased to a notable extent reaching maximum values between 200 and 300 mV, regardless the experimental line considered. Overall, and as has been previously described (Corbella et al., 2014), redox gradient between the anode and the cathode ranged between 400 and 500 mV, regardless the experimental line considered and, thus, no significant differences were recorded among experimental conditions.
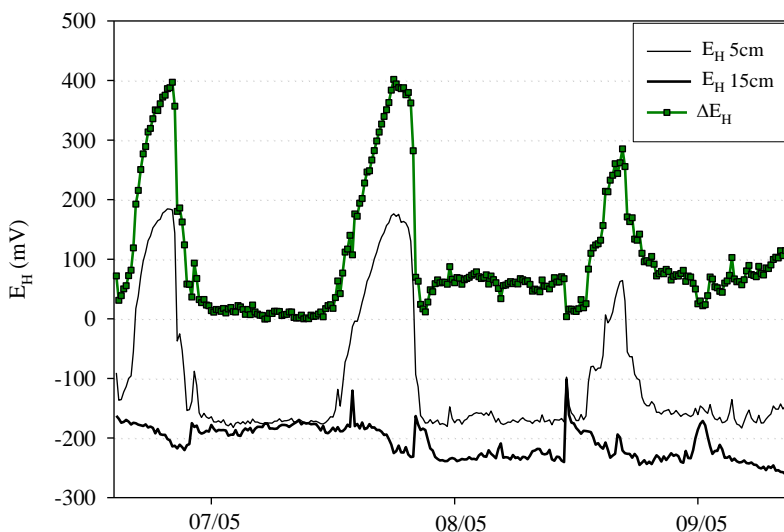
Figure 4.2.3 Representative redox daily pattern for the HUSB line

Concerning cell voltage, MFC replicates performed similarly for both lines (Figure 4.2.4). Furthermore, it is widely accepted that cell electromotive force (E_{emf}) equals the difference between the cathode and the anode potential (redox gradient) minus the losses of the system (namely overpotentials and ohmic losses) (Logan et al., 2006). As expected, voltage recorded followed the same daily pattern than the redox gradient (Figure 4.2.4), showing peacks during sunlight hours and being close to zero during nightly hours. Although there was a notable variability on the peacks, cell voltage started to rise roughly between 11:00 and 15:00h and decreased between 18:00 and 23:00h.
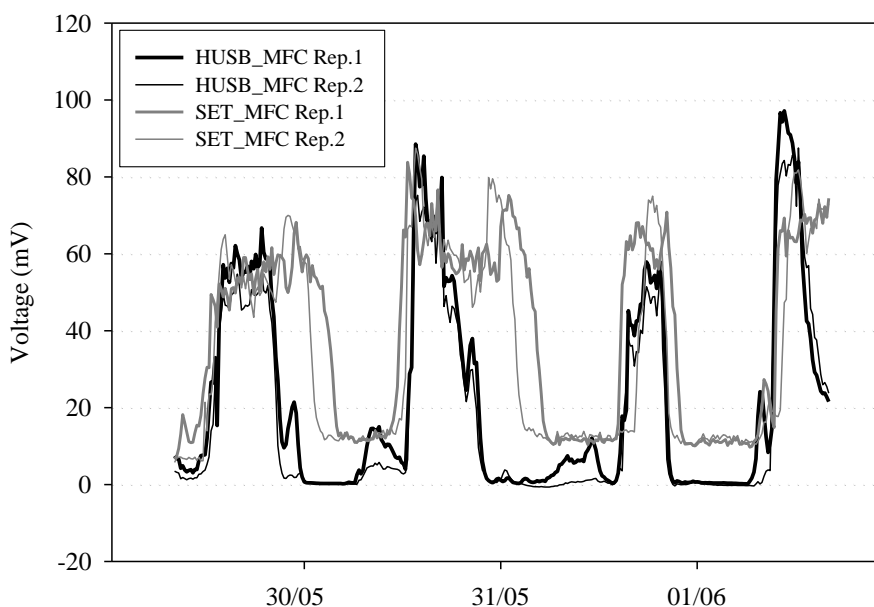
Figure 4.2.4 Representative voltage pattern recorded for microbial fuel cells implemented within the HUSB and the Settler line

During the first period, maximum daily cell voltage averaged 61 ± 29 mV and 50 ± 27 mV for the HUSB and the settler line, respectively. Although the HUSB line achieved a higher maximum cell voltage (136 mV when compared to 103 mV for the settler line), daily average cell voltage, yet higher for the HUSB line, was not significantly different among treatment lines. During the second period, HUSB_MFC achieved a maximum cell voltage of 164 mV.

4.2.3.3 *Effect of evapotranspiration on daily and seasonal cell performance*

As it has been pointed out in the previous section, daily oscillations were observed all through the study period either in terms of redox (Figure 4.2.3) or cell voltage (Figure 4.2.4). Similar patterns have been reported in current literature for MFC implemented in planted environments such as rice paddy fields (De Schamphelaire et al., 2008; Kaku et al., 2008) or, more recently, in constructed wetlands (Villaseñor et al., 2013). So far, some of the authors attribute an increase in the electrical output during sunlight hours to a higher photosynthetic activity of plants that increases the total amount of

substrate available (root exudates) for energy production (De Schamphelaire et al., 2008; Kaku et al., 2008; Strik et al., 2008). Besides, Villaseñor et al. (2013) also reports the positive effect on the cathode of the oxygen released by roots during sunlight hours. However, water losses caused by evapotranspiration in planted systems have been also described to influence MFC performance (Strik et al., 2008). Although water level inside the wetlands was set to be 30 cm, significant water level variations from the design value were observed all along the study period. More precisely, water level within the wetlands decreased from the design level of 3.1 ± 0.9 cm (in May where plants started to grow) to 6.1 ± 1.8 cm (in July where plants were already grown) (Figure 4.2.5). Moreover, intense water level variation, especially from May until the end of the study period, left the cathode of the MFC exposed to the atmosphere during the central hours of the day. When cathode was air-exposed, oxygen availability increased and favoured the current generation due to an increase of the cell voltage.

Accordingly, the potential effects that macrophytes may have had on MFC implemented in constructed wetlands were not detected in this study. Beyond the influence on oxygen availability at the cathode caused by evapotranspiration, any other effect on cell performance such as higher availability of organic matter in the anode from root exudates or higher oxygen content in the cathode by oxygen release from roots was hidden by the predominant cathode limitation. Our results are in accordance with current literature since it is generally accepted that MFC performance is related to oxygen availability at the cathode (Fan et al., 2008; Oh et al., 2004).
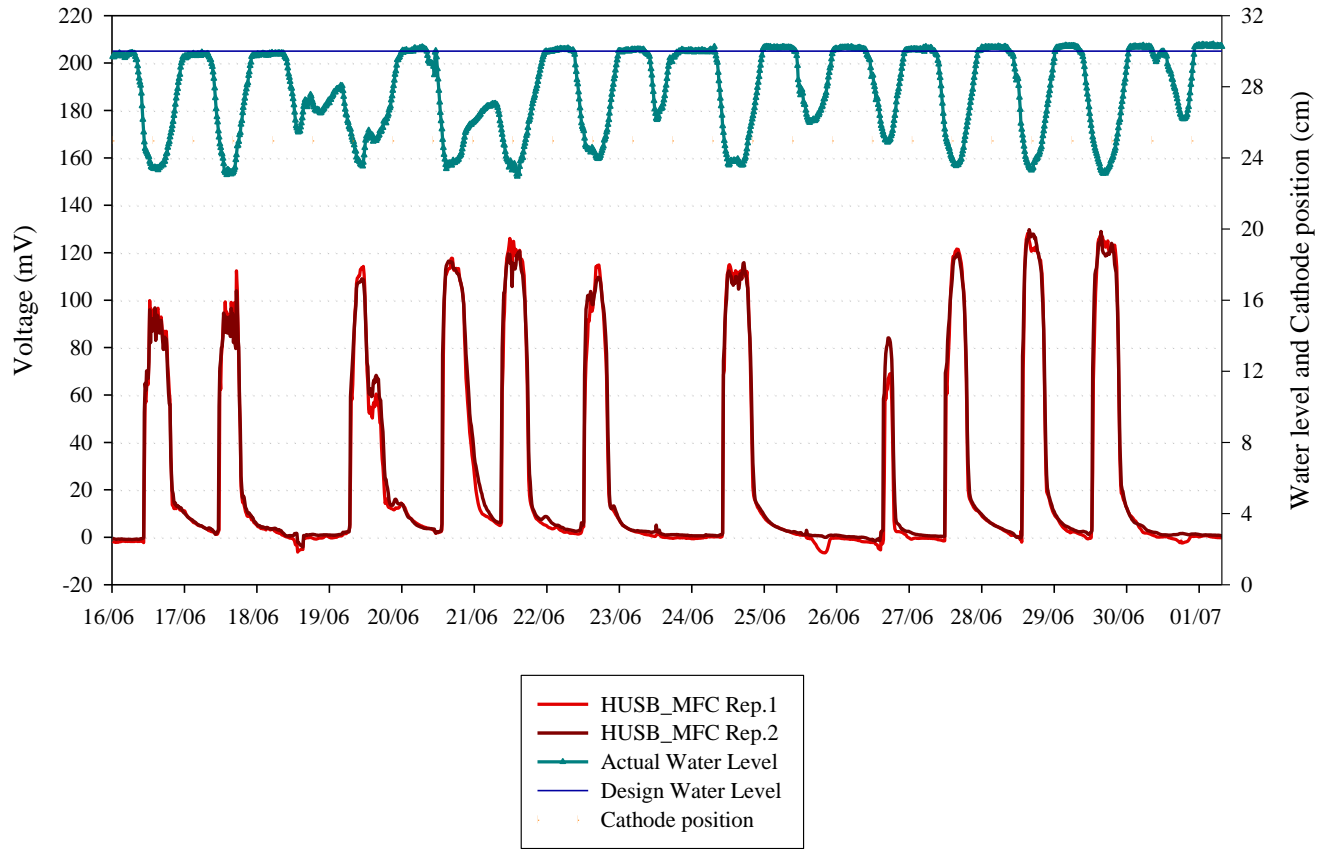
Figure 4.2.5 Representative cell voltage and water level variation for the HUSB line

Furthermore, our results suggested not only that cell voltage was influenced by water level variation on a daily basis, but also in terms of seasonal variations (Figure 4.2.6 A and B). To this regard, from February until mid May, with temperatures of 12.4 ± 4.4 ℃ and plants were not yet developed, no significant power and current were recorded. From middle May until the end of July, when temperature rose up to 21.1 ± 5.1 ℃ and plants were already developed, both current and power density started to increase, reaching maximum values during the first period (both HUSB and settler lines under operation) of 181 mA/m^2 and 25 mW/m^2 and 138 mA/m^2 and 14 mW/m^2 for the HUSB_MFC and the SET_MFC, respectively. During the second period (only the HUSB line) microbial fuel cells achieved the maximum power and current densities recorded for the whole study period (219 mA/m^2 and 36 mW/m^2). Average values for the first period were 82 ± 38 mA/m^2 and 6 ± 5 mW/m^2 and 66±37 mA/m^2 and 4 ± 4 mW/m^2 to the HUSB_MFC and the SET_MFC, respectively. Daily power production values (data not shown) followed also the same pattern as the current and power density, reaching maximum values during the first period of about 259 mWh.m^{-2}.day^{-1} and 158 mWh.m^{-2}.day^{-1} for the HUSB line and the settler line, respectively.

Regarding cell efficiency in the first period, voltage measured (E_{cell}) compared to the maximum attainable (E_{emf}) was, in average, 13 ± 12% and 7 ± 5% to the HUSB and the settler line, respectively. Therefore, results suggested that MFC were highly limited.

Even though no statistical differences were found between lines in terms of current production, HUSB_MFC showed a better performance along both periods. To this regard it could be suggested that the anaerobic nature of this primary treatment may have enhanced the presence of anaerobic microorganisms, such as *Geobacter,* in the system (further addressed). Moreover, the presence of significantly higher concentrations of organic matter in the vicinity of the cells Table 4.2.1) may have also contributed to the higher voltages obtained from the HUSB_MFC.
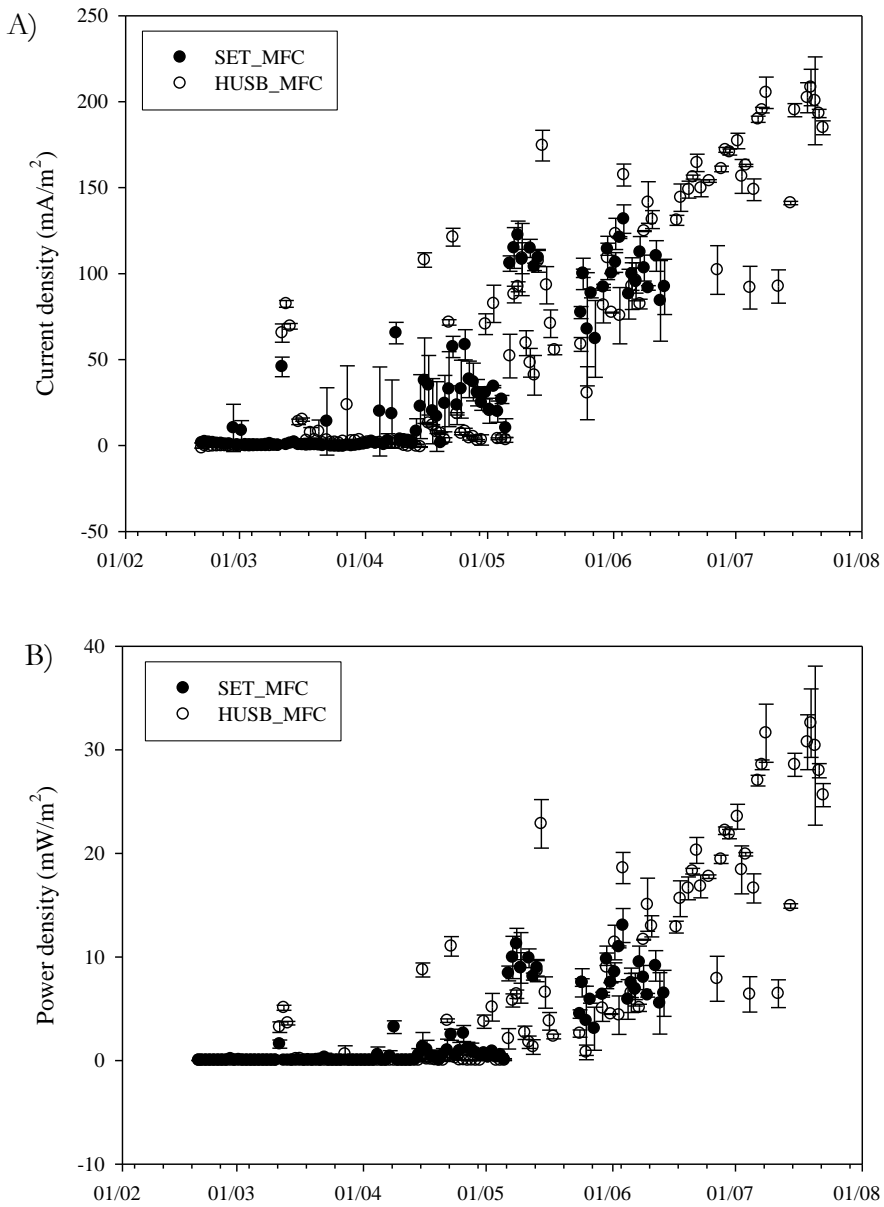
Figure 4.2.6 Daily maximum current (A) and power (B) density evolution along the experiment for the HUSB_MFCs and the SET_MFCs. Note that values from the settler line are only plotted until mid June since after then, only the HUSB line was left in operation

Power and current densities described by Villaseñor et al. (2013) and Yadav et al. (2012) for MFC implemented in constructed wetlands are 43 mW/m² and of 16 mW/m² and 37 mA/m² and 70 mA/m², respectively. Therefore, our results are in the range of that previously reported in literature, though this is the first time that, to the knowledge of the authors, MFC are implemented in pilot-scale wetlands treating real domestic wastewater, where the availability of easy biodegradable substrates is of lesser extent when compared to synthetic wastewater. Overall, despite the HUSB line showed higher maximum power production when compared to the settler line, no significant differences were recorded among treatment lines. It is important to point out that authors believe that one of the reasons behind the lack of significant differences among treatment lines concerning the average cell voltage recorded was the high oxygen limitation at the cathode. Indeed, the experiment on the assessment of cathode limitation conditions performed at the end of the study period confirmed that MFC operated during the whole period of study were probably subjected to a cathode limitation surface (Figure 4.2.7). From Figure 4.2.7 it is clear that in order to avoid any cathode limiting condition the surface of cathode shall be around four times higher than that of the anode.
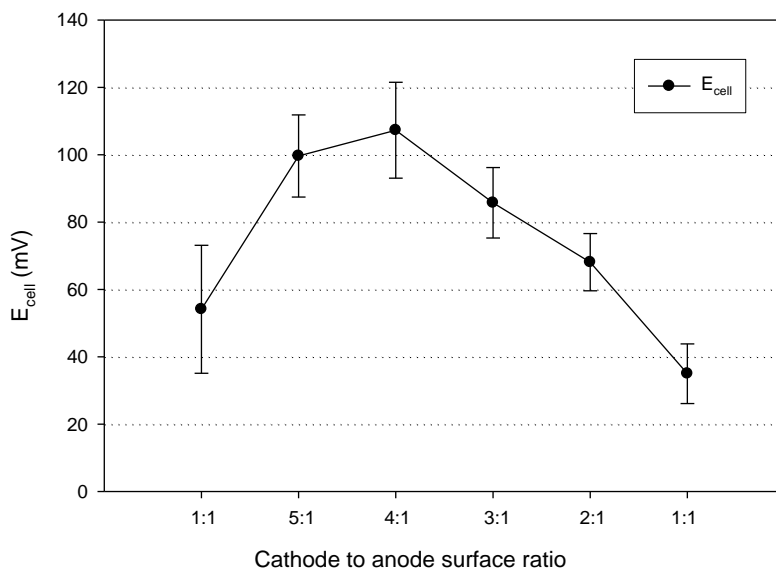
Figure 4.2.7 Influence of cathode to anode surface ratio on E_{cell}. *Note*: error bars are the standard deviation of two replicates; each dot represents a three-day average value of the E_{cell} recorded during sunlight hours

4.2.3.4 Effect of primary treatment on bacterial populations in MFC-implemented CW

Microbial community assessment was conducted on gravel, electrodes (graphite material from open and closed circuit MFC) and from primary treated wastewater from both the settler and HUSB reactor. Samples were taken from early June 2014. Total eubacteria and archaea populations were determined by qPCR and *16S rRNA* gene based 454 pyrotag sequencing approaches.

<u>Total eubacteria and methanogenic populations abundance</u>

Total microbial populations ranged from $2 \cdot 10^8$ to $6 \cdot 10^7$ *16S rRNA* gene copies $\cdot$ mL^{-1}, with methanogens accounting for 0.10-0.13% of total community in both the settler and HUSB-treated wastewater. Total eubacteria

(*16S rRNA* gene) and methanogenic archaea in the effluent of the HUSB reactor were significantly lower (P<0.05) than that of the effluent of the settler (Figure 4.2.S1; Table 4.2.S1). Total eubacterial population recorded for both gravel and electrodes were not significantly different among experimental lines and ranged from $1 \cdot 10^8$ to $3 \cdot 10^8$ *16S rRNA* gene copies $\cdot$ g^{-1}. Methanogenic population ranged from $0.6 \cdot 10^6$ *mcrA* gene copies g^{-1} to $1.2 \cdot 10^6$ *mcrA* gene copies g^{-1} (accounting for 0.22 to 0.49 % of total microbial populations) in graphite samples from active circuits of the settler and HUSB line, respectively. Furthermore, methanogenic populations were significantly higher (P<0.05) in the anode material of MFC operated at closed circuit (active circuit) when compared to gravel, regardless the primary treatment applied. However, only in case of the MFC within the settler line the methanogenic population were significantly different between active and inactive MFC.

16S rRNA gene-based 454 pyrotag sequencing of total eubacteria and archaea

Taking into account the slightly differences recorded regarding total eubacteria and methanogenic archaea abundance, a *16S rRNA* gene-based 454 pyrotag sequencing was carried out to gain insight on microbial community structure of total eubacteria and archaeal populations.

In the present study 16 samples for eubacteria and 14 for archaea were assessed. A total number of 136,925 and 72,233 sequences were obtained for eubacteria and archaea, respectively. After sequence processing, a total high-quality reads of 107,747 and 12,519 were retained for eubacteria and archaea, respectively. The average clean reads for eubacteria per sample were 5,794-8,304 for treated wastewater; 6,652-10,330 for the Settler line samples (gravel and graphite) and 5,020-6,667 reads for the HUSB line samples (gravel and graphite). The coverage (%) ranged from 95.9% to 98.9% for eubacteria and 94.6 to 99.9% for archaea (Table 4.2.S2). However, the average cleaned reads taxonomically assigned as archaea per sample were from five to ten fold lower than those achieved for eubacteria (Table 4.2.S2). The number of OTUs (97% of similarity) for eubacteria ranged from 352 to 434 in wastewater samples, and from 436 to 775 for graphite and gravel samples. The number of high quality reads for eubacteria were not significantly different among experimental lines, regardless the type of sample considered (primary treated

wastewater, gravel, and graphite samples). However, significantly higher diversity was encountered for the HUSB line samples when compared to the settler line based on certain diversity estimators such as OTU numbers (640-775), Chao-1 (837-987), Shannon-Wienner (5.0-5.5), and even a higher evenness index (0.23-0.38) (Figure 4.2.S2 and Table 4.2.S2).

Global diversity results clearly showed the existence of a population shift in MFC implemented in constructed wetlands, specially driven by HUSB pretreated wastewater. The diversity encountered in our 454 *16S rRNA* gene pyrotag libraries in MFC coupled CWs (Shannon index (*H*) in the range of 4.36 to 5.5) was significantly higher than that described elsewhere in constructed wetlands treating domestic wastewater and swine wastewater using the DGGE technique (*H*: 1.1-4 (Calheiros et al., 2009); H: 0.71-1.07 (Dong and Reddy, 2010); tRFLP (*H*: 2.9-3.1) or those using clone libraries (H: 2-3.8) in CW treating industrial wastewater polluted with arsenic and zinc (Arroyo et al., 2013).

Biodiversity of eubacteria (by class) and archaea (by family) in terms of relative incidence for the main taxonomic groups are shown in Figure 4.2.8 A and B (and also in Table 4.2.S3). Settled wastewater showed a high predominance of beta-proteobacteria (average 29%) and Flavobacteria (average 48%) (Figure 4.2.8 A; Table 4.2.S3). Anaerobically pre-treated wastewater showed a predominance of beta and gamma-proteobacteria (average 46% and 32%, respectively) and, to a lesser extent, flavobacteria (6%) and clostridia (7%).

Samples analyzed for eubacteria from the wetland fed with the settler effluent (including gravel and graphite at open and close circuit MFC) presented no significant differences concerning the dominance of groups at class level (Figure 4.2.8 A and B; Table 4.2.S3). Among eubacteria dominant classes were that of alfa-proteobacteria (around 20-32%) and Flavobacteria (around 16-30%).

Samples analyzed for eubacteria from within the wetland fed with the HUSB effluent (including gravel and graphite at open and close circuit MFC) also showed no significant differences concerning the dominance of groups at class level (Figure 4.2.8 A and B; Table 4.2.S3). Among eubacteria dominant

classes were that of Alphaproteobacteria (up to 17%), Deltaproteobacteria (up to 30%); clostridia (up to 18%); bacterioidia (up to ca. 8%); synergistia (up to 9%) and anaerolineae (up to 6%).

A)
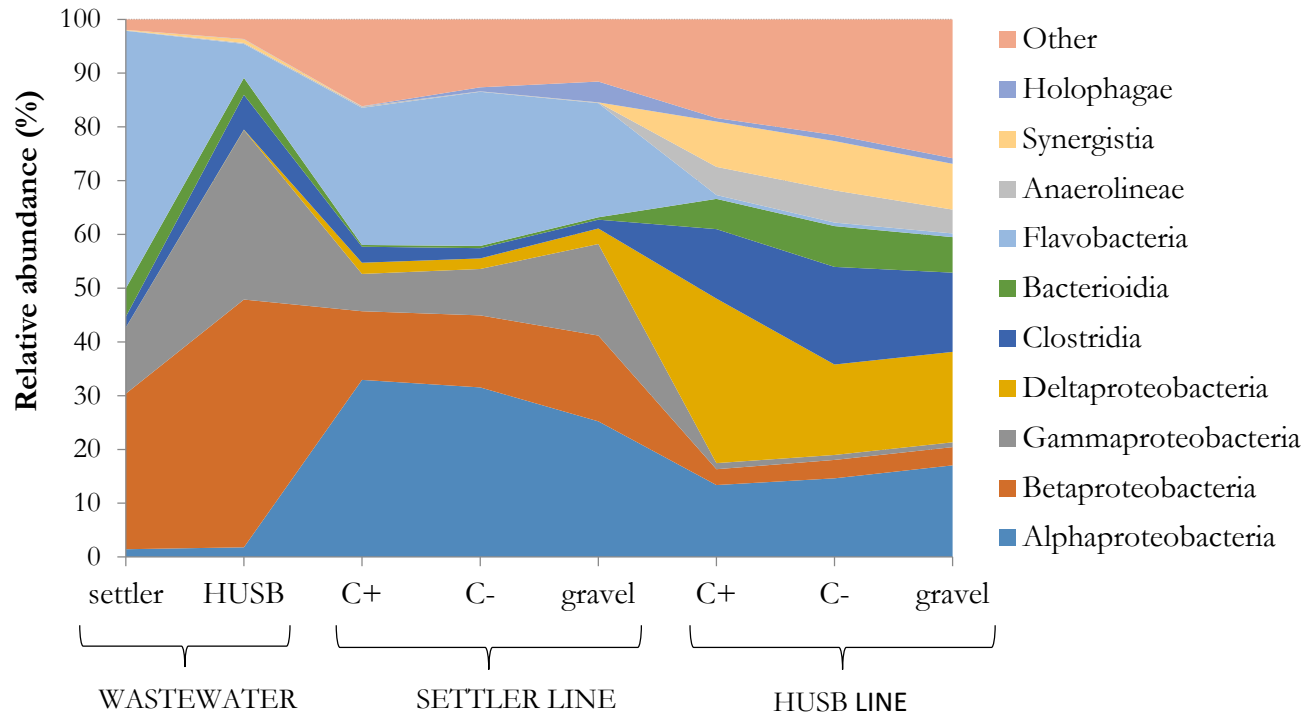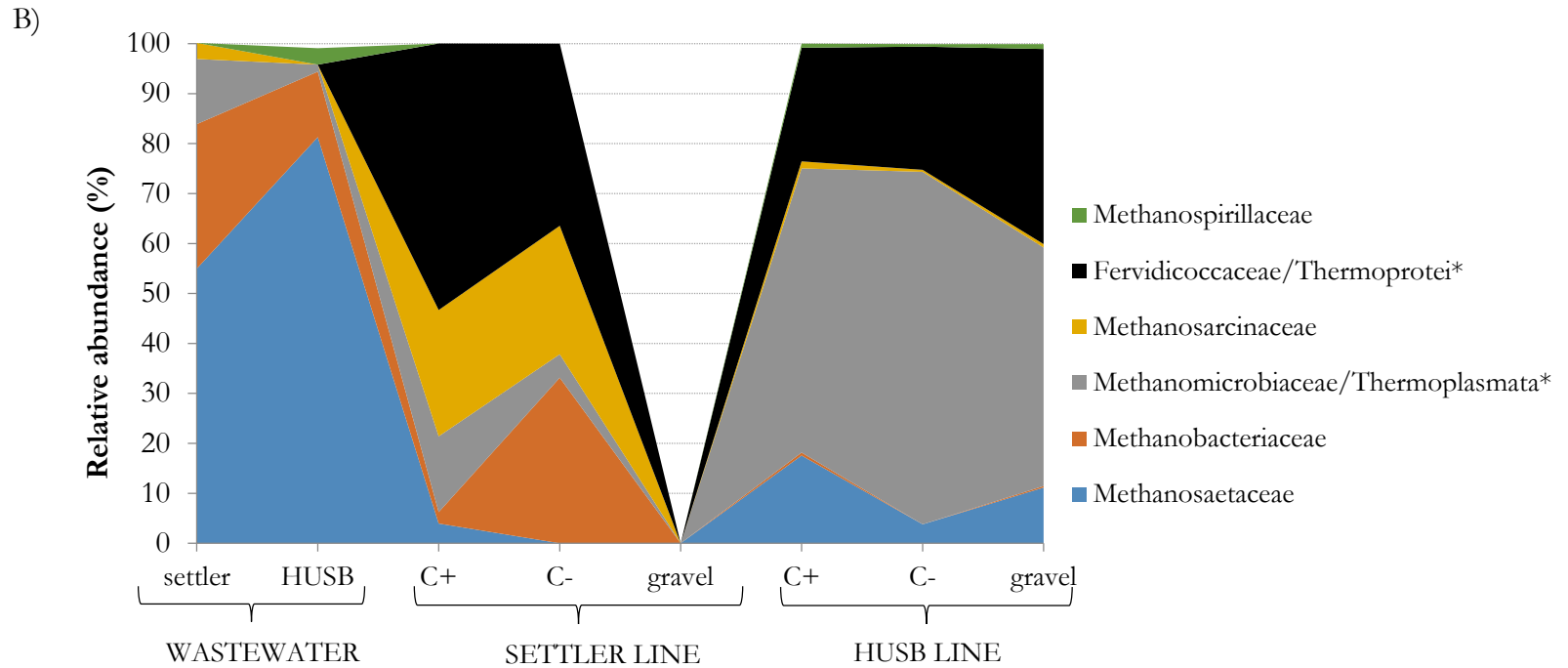
Figure 4.2.8 Biodiversity of main representatives of eubacteria (sorted by class) (A) and archaea (sorted by family) (B) expressed as relative OTUs abundance (%). C+ (graphite in closed circuit); C- (graphite in opened circuit). Thermoprotei class or Fervidicocacceae family in Chrenarchaeota phylum were assigned according RDP Classifier and greengenes respectively. Thermoplasmata class or Methanomicrobiaceae in Euryarchaeota phylum were assigned according RDP Classifier and greengenes respectively. *Note:* Gravel samples from the settler line did not produce any DNA amplification and are not considered in Figure 4.2.8 B

Diversity of eubacteria was significantly higher, encompassing important phylogenetic and quantitative changes, when the HUSB line was compared to the Settler line, regardless the type of sample considered (Figure 4.2.8 A; Table 4.2.S3). More precisely, for gravel and graphite samples of the HUSB line predominant groups were that of clostridia, delta-proteobacteria, Bacteroidia, Synergistia, alfa-proteobacteria and Anaerolineae (Figure 4.2.8; Table 4.2.S3 and 4.2.S4). Accordingly, the fundamental difference in microbial community structure promoted by the two types of primary treatment here considered was the high enrichment in Bacteroidia (OTUs 1 and 2) and delta-proteobacteria class in the gravel and graphite samples of the HUSB line (OTU 4) when compared to gravel and graphite samples of the settler line.

Regarding delta-proteobacteria class it is remarkable the relative predominance of the Geobacteraceae family in gravel and graphite samples of the HUSB line (19% and 5% of relative abundance for the graphite of active MFC and gravel samples, respectively). Within the Geobacteraceae family it is of special interest the high relative abundance of one OTU belonging to *Geobacter* in active MFC of the HUSB line (from 13 to 16%). In the case of gravel and graphite samples from the settler line, Geobacteraceae were not only less favored (below 2%), but even the detected Geobacteraceae OTUs were different from that of the OTUs found in samples from the HUSB line (Table 4.2.S5). Promoting hydrolytic conditions in the HUSB line, the establishment of anaerobic conditions was favoured in wastewater inflow. Therefore, HUSB pre-treatment could favour *Geobacter* enrichment and activity outcompeting certain microbial populations on gravel and anode material in MFC-CW.

Regarding archaeal population, it is noteworthy the high relative prevalence of Methanosaetaceae family at the effluent of both types of primary treatments (55% and 81% for the settler and HUSB reactor, respectively). Furthermore, there was a shift in methanogenic archaea that consisted in a high decrease of Methanosaetaceae encompassed by an enrichment of Methanomicrobiaceae/Thermoplasmata (OTU2) as it is assigned by Greengenes/RDP Bayesian Classifier in HUSB line. In addition, a non-methanogenic phylum (Chrenarchaeaota) was highly predominant in gravel and graphite samples from both experimental lines. (Chrenarchaeota, assigned as Fervidicoccaceae/Thermoprotei by Greengenes/RDP Bayesian

Classifier) (Wang et al., 2007) (37-53% and 23-39% to the settler and HUSB lines, respectively).

Multivariate statistical analyses were conducted by means of covariate-principal component analyses (PCA) (Figure 4.2.9 a and b, and Figure 4.2.S3-4.2.S4) and correspondence analyses (CA) with similar results. PCA and CA analysis revealed the existence of three main separate groups of samples encompassing different microbial communities (primary treated wastewater samples, settler line and HUSB line) (Figure 4.2.9 a and b). Regarding eubacteria, the main OTUs with the higher component weight/contribution in wastewater were OTUs 8 and 673, closely similar to *Comamonas denitrificans* (beta-proteobacteria) and OTUs 10 and 224 belonging to well-known fermentative *Acinetobacter* genus (gamma-protebacteria). Regarding the settler line, OTUs belonging to *Cloacibacterium* (Bacteroidetes, OTU 1), *Phenylobacterium* (alpha-proteobacteria, OTU 3) and *Sphingopixis* (alpha-proteobacteria, OTU 7) were the main OTUs to define the group. For the HUSB line it is worth mentioning the presence of two main distinctive OTUs on PCA/CA biplot (Figure 4.2.9 a and b and Figure 4.2.S3-4.2.S4), OTU 2 belonging to *Rhodobacter/Bacteroidetes* (assigned by Greengenes/RDP Bayesian classifier databases, and OTU 4, closely similar in sequence (99.7%) to an environmental *Geobacter* (delta-proteobacteria) and to *Geobacter lovleyi* Geo 7.1A (97.16%). OTU 4 was not detected neither in primary treated wastewater nor in the settler line, and was clearly more enriched in anode under closed (active) circuit (13-16%) for the HUSB line than in gravel (3%), and almost absent (0.6-1.5%) in opened (inactive) circuit (Table 4.2.S3). Taking into account that MFC within the HUSB line tended to show higher current and power densities when compared to the settler line, OTU 4 related with *Geobacter* might be a good candidate as a key player for exoelectrogenic and current production in this system. Coincidently, a *Geobacter* enrichment was also reported in constructed wetlands treating 1,2-dichloroethene-contaminated groundwater (Imfeld et al., 2010), and recently in the anode of lab-scale MFC coupled to a constructed wetland system for decolorization of azo dyes (Fang et al., 2013). However, contrarily to Fang et al. (2013), in the present study methanogenic archaea belonging to *Methanosaeta* has been just slightly enriched in the anode material of both experimental lines (Table 4.2.S5). Current research is revealing the occurrence of exoelectrogenic activity in

Methanosarcina and *Methanosaeta* sharing electrons with a concomitant *Geobacter*, (Rotaru et al., 2014a, 2014b) promoting potential electron current production in MFC and complex microbial communities such as those harboured in natural environments.
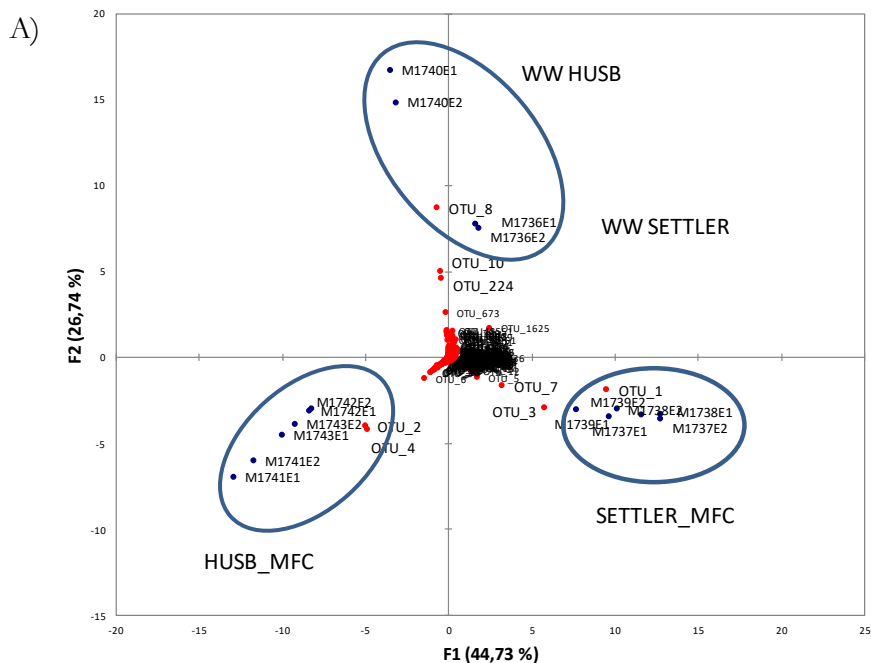
Figure 4.2.9 - A Covariance-based Principal Component Analysis biplot of A) eubacterial and B) archaeal OTUs distribution from pyrosequencing analysis from different samples
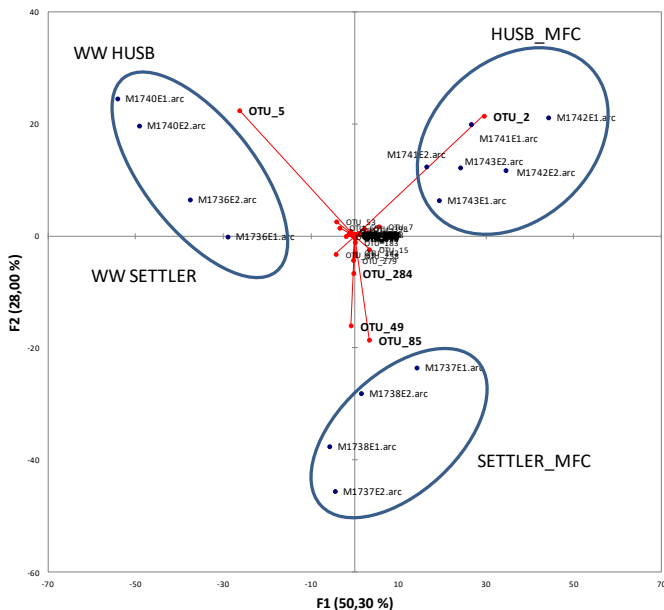
Figure 4.2.9 - B Covariance-based Principal Component Analysis biplot of A) eubacterial and B) archaeal OTUs distribution from pyrosequencing analysis from different samples

4.2.4 Conclusions

- Maximum current and power densities recorded were 219 mA/m^2 and 36 mW/m^2.

- Redox gradient between electrodes and cell voltage followed a very conservative pattern along the day with higher output cell voltage values during daylight hours.

- The main parameter controlling the cell voltage was water level variation within the wetlands that resulted from intense evapotranspiration which exposed the cathode to air.

- Microbial fuel cells implemented in a constructed wetland fed with the effluent of an anerobic digester showed a high predominance (13-16% of relative abundance) of one OTU belonging to *Geobacter* that was absent for the settler line MFC.

Long-term assessment of best cathode position to maximise microbial fuel cell performance in horizontal subsurface flow constructed wetlands

Abstract

The cathode of microbial fuel cells (MFC) implemented in constructed wetlands (CW) is generally placed at the water surface to provide a rich oxygen environment. However, water level variations caused by plants evapotranspiration in CWs might decrease MFC performance by limiting oxygen transfer to the cathode. Main objective of this work was to quantify the effect of water level variation on MFC performance implemented in HSSF CW. For the purpose of this work two MFCs were implemented within a HSSF CW pilot plant fed with primary treated domestic wastewater. Cell voltage (E_{cell}) and the relative distance between the cathode and the water level were recorded for one year. Results showed that E_{cell} was greatly influenced by the relative distance between the cathode and the water level, giving an optimal cathode position of about 1 to 2 cm above water level. Both water level variation and E_{cell} were daily and seasonal dependent, showing a pronounced day/night variation during warm periods and showing almost no daily variation during cold periods. Energy production under pronounced daily water level variation was 40% lower (80 ± 56 mWh/m²·day) than under low water level variation (131 ± 61 mWh/m²·day). Main conclusion of the present work is that of the performance of MFC implemented in HSSF CW is highly dependent on plants evapotranspiration. Therefore, MFC that are to be implemented in constructed wetlands shall be designed to be able to cope with pronounced water level variations.

CW-MFC implemented in HSSF CW

CW-MFC

water level

cathode 2nd period →

cathode 1rt period →

anode →

gravel →

10 cm

10 cm

HSSF CW PILOT PLANT
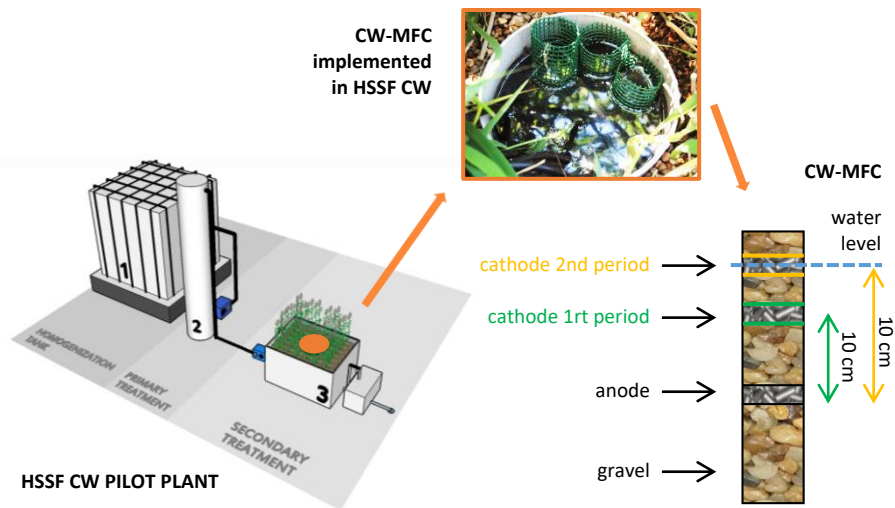
4.3.1 Introduction

Microbial Fuel Cells (MFCs) are bioelectrochemical systems that generate electricity from organic matter oxidation using bacteria as catalysts (Logan, 2008). Electrons produced during the oxidation are transferred to the electrode (anode) from where they flow through a conductive material and a resistor to reduce an electron acceptor at the cathode (Logan et al., 2006; Rabaey and Verstraete, 2005). The current generation in MFCs depends on the redox gradient between the anode and cathode. For MFC to produce an electric current two areas are required, one under reduced redox conditions where organic matter is oxidized and the other one under higher redox potential where terminal electron acceptors are reduced. Horizontal subsurface flow constructed wetlands (HSSF CWs) are engineered treatment basins filled up with granular media and planted with macrophytes that are used mainly for the treatment of domestic wastewater. In HSSF CWs the presence of both organic matter and naturally generated redox gradients can be exploited to produce energy via MFCs (Corbella et al., 2015a, 2014). The implementation of MFCs in CWs (and in particular on HSSF CW) is in its first stage and current scientific information available on the topic is limited. So far, some studies have been developed in pilot-scale systems (Doherty et al., 2015; Villaseñor et al., 2013), though most of them were based on laboratory-scale experimental designs (Fang et al., 2013; Yadav et al., 2012; Zhang et al., 2013) and used synthetic wastewater instead of real domestic wastewater. In HSSF CWs, redox potential decreases with depth generating a

vertical redox gradient between the upper layer, which is in higher redox conditions, and the deeper layers where anaerobic environment predominates (Dušek et al., 2008; García et al., 2003; Pedescoll et al., 2013a). Main configuration for MFC implementation in HSSF CWs relies on setting a cathode at the surface of the system while the anode remains buried in the deeper zone of the treatment bed. Evapotranspiration caused by plants induces marked daily variations on water level within the treatment bed and therefore, has a notable influence on wetland's redox conditions (Mann and Wetzel, 1999; Pedescoll et al., 2013a). Notable fluctuations of water table caused by plants evapotranspiration may vary MFC performance on daily and seasonal terms by changing the availability of oxygen at the cathode. Consistent with this, cathode has been considered to be one of the major sources of limitation in CW-MFCs due to the slow kinetics of oxygen reduction and the scarcity of oxygen in CW environment (Corbella et al., 2015a; Doherty et al., 2015). However, although most of the reported CW-MFCs include plants in the experimental designs, none of them consider the effect of evapotranspiration on cathode performance (Fang et al., 2013; Villaseñor et al., 2013). Up to date daily fluctuations of CW-MFC performance have been attributed to the photosynthetic activity of plants (Liu et al., 2014; Villaseñor et al., 2013) with no specific mention to water level variation caused by plants evapotranspiration. Therefore, the purpose of this study was to determine the influence of water level fluctuations on the performance of MFCs implemented in HSSF CW that, as far as authors know, is currently unaddressed. To this purpose the relative distance between cathode and water level was continuously monitored for one year in two MFCs implemented in HSSF CW pilot plant fed with real domestic wastewater. The contribution of macrophytes to the water table variation and the effect of the distance between electrodes was also studied. Results shown in this study provide useful information to optimise the architecture of microbial fuel cells that are to be implemented in HSSF CWs.

4.3.2   Materials and methods

4.3.2.1   Pilot plant description

The pilot plant was located in Barcelona and it was set up in March 2011. It consisted of one wetland of 0.4 m² of surface with a gravel matrix

having an initial porosity of about 40% and a depth of 35 cm (Figure 4.3.1). Water level inside the wetland was kept at about 30 cm depth (5 cm below the gravel surface). The wetland was planted with common reed (*Phragmites Australis*) and was very mature by the time this work was carried out (year 2013). The wetland had a sampling area of 20 cm diameter located in the centre of its surface. This area was not filled with gravel and was used to take samples and to place the probes and microbial fuel cells.

The wetland was fed with urban wastewater which was pumped directly from the municipal sewer. Initially wastewater was coarsely screened and after that it was stored in a 1.2 m³ tank of five hours of hydraulic retention time (HRT) before being conveyed to the primary treatment. In the tank, wastewater was continuously stirred to avoid solids sedimentation. Primary treatment consisted of a hydrolytic up-flow sludge blanket reactor (HUSB reactor) with a total volume of 115 L that was operated at four hours of HRT. Secondary treatment consisted of one horizontal sub-surface flow constructed wetland fed under a continuous flow of 0.875 L/h (design HRT was that of 2.6 days).
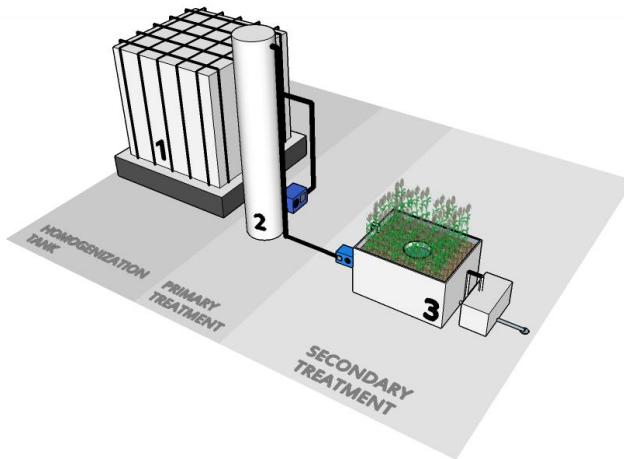
Figure 4.3.1 Constructed wetlands pilot plant. First picture: the wetland during summer 2013. Second figure: a scheme of the pilot plant process line

4.3.2.2 *Wastewater physical and chemical analysis*

Water quality parameters (total and soluble chemical oxygen demand (COD) and ammonia) were measured at the influent, middle part, and effluent of the wetland. They were analysed once every week or two weeks according to Standard Methods (APHA, 2005). The influent and effluent flow was daily monitored which allowed us to calculate evapotranspiration rates and removal

efficiencies on a mass balance basis. Air temperature was obtained from a nearby meteorological station (Department of Astronomy and Meteorology, University of Barcelona).

4.3.2.3   MFC's configuration and monitoring

Three MFC were implemented within the HSSF CW pilot plant. Two MFC were operated at closed circuit and one was left at open circuit condition. For the purpose of this work only the two MFC working under closed circuit will be considered. MFCs implemented consisted of a plastic mesh of 40 cm length and 5 cm diameter filled with 35 cm of gravel in order to simulate wetlands' characteristics (Figure 4.3.2). The anode and cathode were made of twenty cylindrical graphite rods (1 cm length and 0.6 cm diameter) each, covered by a mesh of stainless steel (marine grade 316L) (Figure 4.3.2). They were cubic shaped with a projected surface of 7.5 cm² and 1 cm high. The external circuit was closed by connecting both electrodes with one external resistance of 1000 ohms by means of cooper wires. Epoxy materials were used to preserve metal connections from wastewater corrosion. MFC were placed in the central part of the treatment bed which was empty of gravel (Figure 4.3.2).
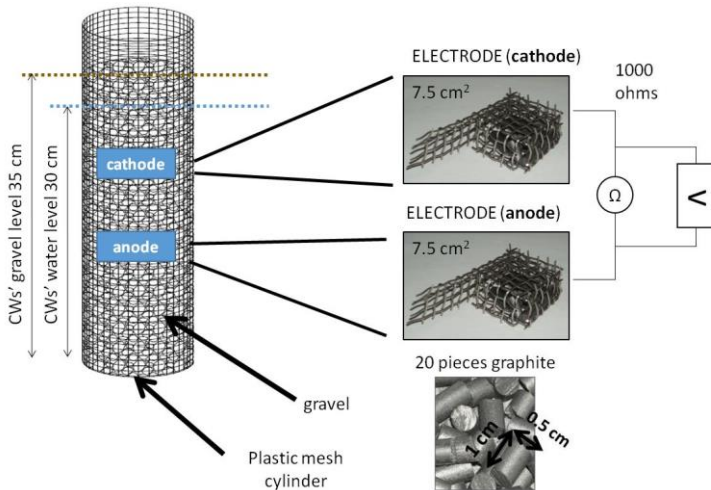
Figure 4.3.2 MFC experimental set up. The upper picture shows MFC implemented within the wetland. The bottom picture shows the schematics of MFC and the picture of the electrodes implemented

MFCs were monitored during two consecutive periods of six months each in which two different cathode positions were tested. Different cathode positions were applied in order to get a wider range of the relative distance between cathode and water level. During the first period (from February to July 2013) the cathode was placed 5 cm below the design water level. During the second study period (from August 2013 to January 2014), the cathode was placed at the same level as the design water level. In both cases, anodes were located 10 cm below cathodes (15 cm and 10 cm below the design water level, respectively).

MFCs were connected to a datalogger (Datataker DT50 series 3) which collected a value of voltage across the external resistance every 15 minutes. Current was calculated following ohms' law and power calculated by

means of $P=V^2/R$, where P represents power, V is voltage and R regards the external resistance. Current and power density values were related to the projected anodic area, which was considered to be the base of the electrode (7.5 cm^2) in order to be able to express energy or power production per wetland surface.

4.3.2.4   Macrophytes effect of MFC performance

The pilot plant consisted of four SSF CW of 0.4 m^2 depth planted with common reed (*Phragmites australis*) treating primary treated domestic wastewater. Two wetlands were operated at 14 g COD/m2.day and two at 41 g COD/m2.day. Two MFCs were implemented within each wetland. MFCs electrodes consisted of graphite rods of 0.5 cm diameter and 1 cm length wrapped in stainless steel mesh marine grade 316L (7.5 cm2 of projected surface area). Electrodes were connected by epoxy sealed wires and the circuit was closed using a 1000Ω external resistance. Cell voltage across the external resistance was measured every 15 min using a data logger. The effect of macrophytes on power production was evaluated for 7 days by covering two of the wetlands (one operated at high and the other at low organic loading) using a synthetic fabric which allowed air exchange but prevented 98% of light penetration.

4.3.2.5   Effect of the distance between electrodes: lab-scale and pilot plant CW-MFCs

The effect of the distance between electrodes on CW-MFCs production was addressed in a lab-scale MFC. The MFC consisted of two chambers (the cathodic and anodic chambers). The anodic chamber consisted of a PVC cylinder of 9 cm diameter and 30 cm high filled up with gravel (D_{60}=7.3; Cu=0.8; porosity=40%) and wrapped in stainless steel mesh (marine grade 316 L, 5 mm space between wires and 1 mm wire diameter). The anode camber of the MFC was designed to simulate a core of a shallow wetland gravel bed where the electrons derived from exoelectrogens organic matter oxidation would be transferred to the electron collector (stainless steel mesh). In order to ensure adequate mixing conditions, water inside the anode chamber was continuously recirculated by means of a peristaltic pump (Damova MP-3035-6M; Toshiba VF-nC3). The cathode chamber consisted

of a PVC cylinder placed just above the anode chamber filled up with 5 pieces of graphite felt (Alfa Aesar, 1.12 cm thick, 99.9%; metal basis) of 60 cm^2 each inter-connected using stainless steel wires (marine grade 316L). A layer of glass wool was placed between the anode and the cathode chamber so as to avoid any oxygen leaking from the cathode (Venkata Mohan et al., 2008). The anode and the cathode were externally connected by means of copper wires through an external resistance of 1000 ohms. MFCs were operated under MFC mode thus no external energy was provided. Finally, voltages generated were measured across the external resistance and stored every 5 minutes by means of a datalogger (Campbell Scientific CR1000).

Lab-scale CW-MFCs were operated under batch mode. Every 24 hours, systems were fed with real wastewater and the distance between the anode and the cathode was increased by one step (see Figure 4.3.3). Distances tested were 10, 12.5, 15, 17.5 and 20 cm. Voltages were recorded all along the experiment and average voltages corresponding to each distance were calculated from voltages recorded over 22.3±2.8 h.
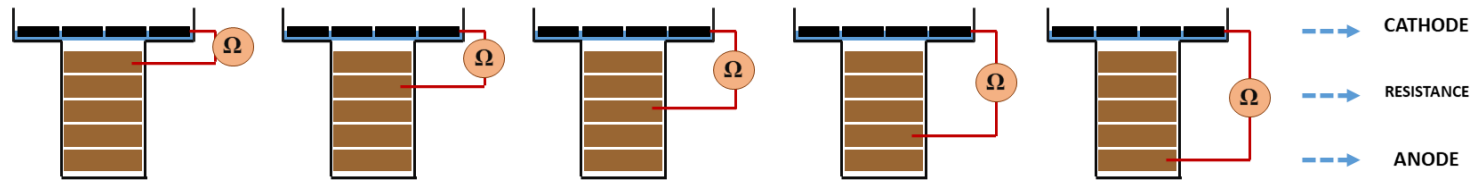
Figure 4.3.3 Scheme of the lab-scale CW-MFC used in the experiment and the sequence followed to determine the effect of the electrode distance on cells' performance

4.3.2.6 *Statistical analyses*

Differences among experimental conditions for any of the considered parameters were determined by carrying out an ANOVA test, T-tests and Wilcoxon tests depending on the type of dataset considered. Data normality and homogeneity of variances were determined by performing the Kolmogorov-Smirnoff and Levenne tests, respectively. Differences among experimental conditions were considered significant at p values below 0.05. All statistical analyses were performed using the software package R 3.0.2.

4.3.3 Results and discussion

4.3.3.1 *Plant treatment performance and organic matter available for MFC functioning*

Soluble and total COD and ammonia were surveyed at the inlet, middle and outlet of the wetland during both periods. As shown in Table 4.3.1, despite the high variability of results, the organic matter concentration at the inlet in terms of both soluble and total COD, was higher during the first period than during the second one (p value<0.05). However, no statistical differences were found between removal efficiencies in both periods. Accordingly, removal efficiencies for total COD were 61±19% and 60±10% during the first and second period, respectively. COD removal efficiencies were slightly lower than those found in the literature, where reported values range from 65 to 80 % (Puigagut et al., 2007b). However, it is worth mentioning that organic loading in our pilot plant (ca. 15 g COD. m^{-2}.day^{-1}) was slightly higher than that generally recommended for HSSF CW (Kadlec and Wallace, 2009). The amount of fuel for MFC functioning is of capital importance for MFC performance (Liu et al., 2004). In our study organic matter concentrations in the vicinity of the MFC were similar during both study periods (ca. 140±50 mg/L and ca. 90±30 mg/L for total and soluble COD, respectively for the first study period and ca. 170±70 mg/L and ca. 120±30 mg/L for total and soluble COD, respectively for the second study period). Finally, it is worth mentioning that the effect that

MFC might have exerted on wastewater treatment efficiency will not be considered in the present work since MFC active electrodes represented a very small proportion ($\approx 0.02\%$) of the total treatment bed volume.

Table 4.3.1 Total and soluble chemical oxygen demand (COD) and ammonia concentrations at inlet, middle and outlet of the wetland during the first and second period. Note: average values are based on n=16 per experimental period.

			FIRST PERIOD			SECOND PERIOD		
			in	middle	out	in	middle	out
COD	Total	$mg\ O_2/L$	323±33	137±53	126±61	254±94	175±70	99±26
	Soluble	$mg\ O_2/L$	178±51	88±29	96±43	132±58	122±33	80±24
Ammonia		$mg\ NH_4\text{-}N/L$	41±7	-	19±19	29±7	-	24±18

In terms of ammonia, again, inlet ammonia concentration was higher during the first period than during the second one (p value<0.05). Both periods were six months each and, therefore, the performance of the pilot plant was affected by seasonal variations. It is reported that nitrogen removal is influenced by temperature (Vymazal, 2007) leading to larger removal efficiencies achieved during warmer periods. This behaviour was especially marked during the first study period. Thus, the average removal efficiency during the whole period was 60±40%, but the mean value obtained from February to mid-May was 29±24% and from mid-May to end of July 98±2%.

Temperature is described to affect HSSF CW microbial processes and its treatment performance (García et al., 2010). CW-MFCs function on a microbial basis and therefore, as in conventional MFCs, temperature may also affect their performance (Jadhav and Ghangrekar, 2009). Accordingly, meteorological data from both periods was analysed to ensure they were comparable in terms of temperature. Results showed very similar average temperatures during the first and the second periods (16±6 and 17±6 °C, respectively) thus being comparable despite the fact that both experimental conditions were tested during different periods (February-July and August-January) (Figure 4.3.S1).

4.3.3.2 Daily cell voltage pattern

Figure 4.3.4 A and B depict a representative cell voltage pattern recorded under intense evapotranspiration during the first and second experimental periods, respectively. Figure 4.3.5 depicts a representative voltage pattern recorded under low evapotranspiration conditions during the second experimental period. It is worth mentioning that during the first experimental period, where the cathode was set at 5 cm below the design water level, cell voltage was close to zero for the most part of the day during cold periods (low evapotranspiration conditions due to the absence of plants). Figure 4.3.4 A and B show that cell voltage was dependent on the water level variation along the day. During the first study period (where cathode was located 5 cm below water level) higher MFC performances were generally recorded from noon to about 6 pm (Figure 4.3.4 A) when cathode was exposed to the atmosphere due to a lower water level caused by evapotranspiration. During the second experimental period (where cathode

was located at water level) higher MFC performances were recorded during hours were evapotranspiration was not intense (Figure 4.3.4 B). Accordingly, cell voltages remained high during most part of the night and dropped during daylight when the cathode was well above the water level and reached a drying state that hampered any electrons flow (short-circuit conditions). Our results on cell voltage pattern are in accordance to those previously stated in current literature were marked daily oscillations of MFC voltage were recorded (Villaseñor et al., 2013). However, daily cell voltage oscillation of MFC implemented in wetlands has been attributed so far to the photosynthetic activity of plants (Doherty et al., 2015; Villaseñor et al., 2013). Our results suggest that, even though carbon exudates are a good carbon source for powering a MFC (De Schamphelaire et al., 2008), the main factor governing the cell voltage in our system was the availability of oxygen at the cathode which was related to water level variation caused by plants evapotranspiration.
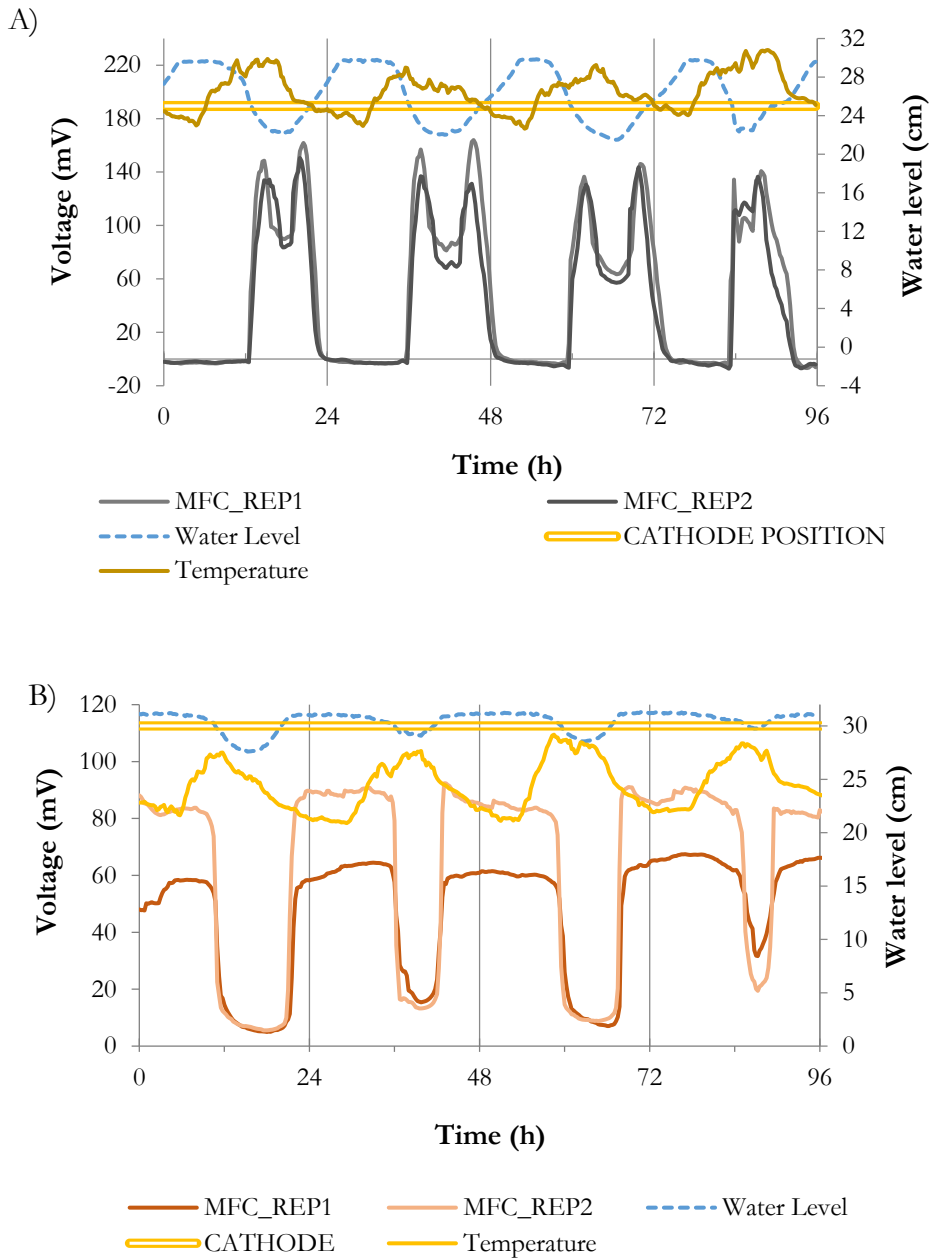
Figure 4.3.4 Representative cell voltage patterns (two replicates, REP1 and REP2) recorded during high evapotranspiration conditions for the first experimental period (A) and second experimental period (B). Note: negative cell voltage values are the result of small inaccuracies of the measurement equipment
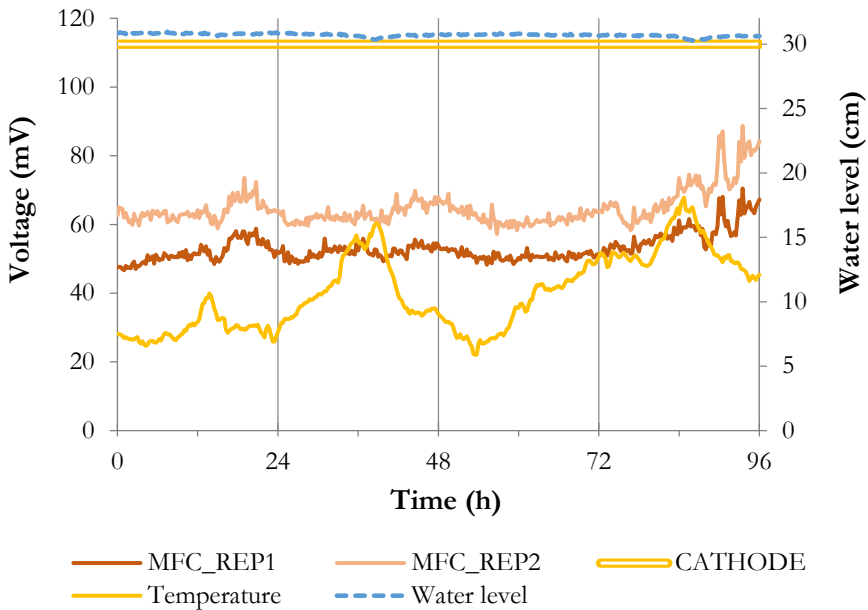
Figure 4.3.5 Representative cell voltage patterns (two replicates, REP1 and REP2) recorded under low evapotranspiration conditions for the second experimental period

4.3.3.3 Best cathode position to maximise MFC performance

Figure 4.3.6 depicts the cell voltage recorded along the one-year study period against the relative distance between cathode and the water level. Results show that, both during the first and the second period, only when the cathode was slightly above water level (position around 1 to 2 cm above water level) cell voltage was maximised. These results suggested that, even though it is reported that the upper water layer of HSSF CWs is under higher redox conditions (García et al., 2003; Pedescoll et al., 2013a), the real oxidized layer from which we can benefit by implementing MFC is of very little extend. Consequently, when cathode was even slightly submerged there was not enough redox gradient between electrodes (anode-cathode) for MFC to produce any significant current. Performance of cathodes is therefore, considered for the authors of this work as the main source for MFC performance limitation. Our results are in accordance to previous reports on in conventional MFCs, where cathodes have been described as the main

limiting factor of microbial fuel cell performance (Ahn et al., 2014; Fan et al., 2008). Actually, it has been demonstrated that power generation is dependent on oxygen availability at the cathode (Ahn et al., 2014; Ferreira-Aparicio and Chaparro, 2014; Oh et al., 2004; Zhang et al., 2013) and also that air cathodes, compared to aqueous ones, lead to higher cell output not only due to higher oxygen concentrations but also to higher mass transfer rates (Fan et al., 2008). Furthermore, in conventional MFCs, cathodes' flooding (Ferreira-Aparicio and Chaparro, 2014; Zhang et al., 2013), air humidity and water pressure (Ahn et al., 2014) have been described to be factors affecting oxygen accessibility. Considering all that, the authors believe that in order to avoid cathode limitation in MFC implemented in CW, cell architecture shall address the possibility to cope with intense water level variations. To this regard, authors believe that a cathode based on a thick layer of graphite placed at the upper part of the treatment bed will allow the CW-MFC to have always an active cathode zone for oxygen reduction, regardless the water level within the wetland. According to our results, water level can vary up 10 cm from the design water level (Figure 4.3.4 A; Figure 4.3.6). Therefore, the thickness of the graphite layer should be, at least, 10 cm.
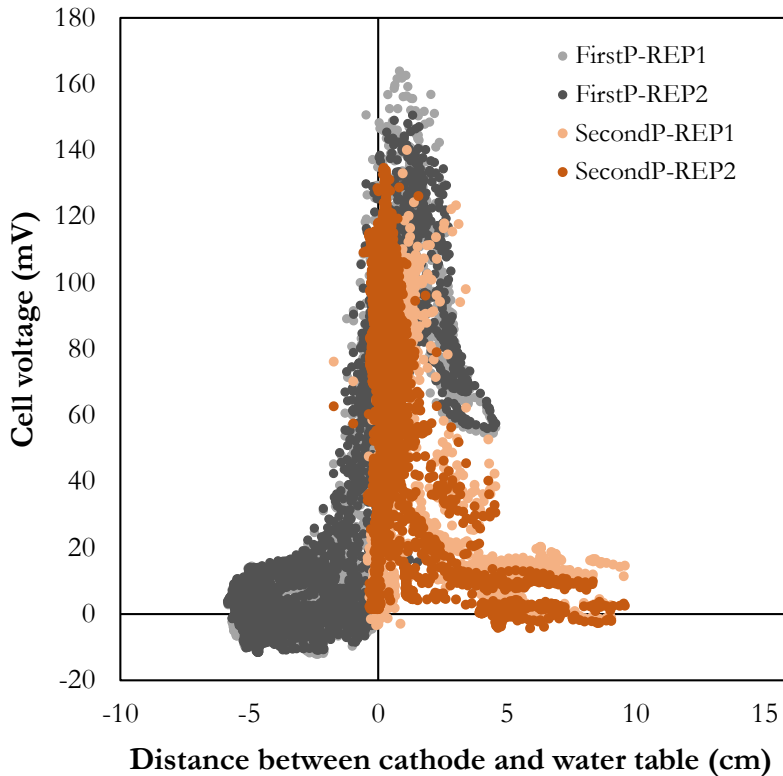
Figure 4.3.6 Voltage plotted against the relative distance between cathode and water table from both replicates (REP1 and REP2). *Note¹*: Negative values of relative distance between cathode and water level means that the cathode is below water level. *Note²*: negative cell voltage values are the result of small inaccuracies of the measurement equipment

4.3.3.4 Effect of water level variation on cell voltage and energy production

Cell voltage and energy production were assessed by comparing MFC performance under conditions of low water level variation (LWL) and high water level variation (HWL), regardless the study period considered. We want to point out that, as mentioned before, cell voltage along the day during cold months of the first study period the MFC was close to zero. Therefore, the data will not be considered for the purposes of this section. Voltage and energy produced under LWL conditions was calculated from the cold months of the second experimental period. Results showed that maximum cell voltage

values were recorded for MFC working under HWL conditions. Accordingly, under very intense evapotranspiration conditions most of the cell voltages recorded achieved values higher than 120 mV, while during the LWL these figures were rarely achieved. However, when daily maximums were statistically compared, no significant differences could be detected.

Contrary to voltages generated, energy produced (mWh/m^2·day) was significantly lower (p<0.05) under HWL conditions when compared to LWL conditions. More precisely, energy produced under HWL conditions was, in average, 40% lower (80±56 mWh/m^2·day) than under LWL conditions (131±61 mWh/m^2·day) (Figure 4.3.7). This result is because MFC operated under higher water level variations resulted in periods of high cell voltage and periods were cell voltage remained essentially constant and close to zero. On the contrary, MFC operated under low water level variations resulted in a nearly constant cell voltage signal along the day.
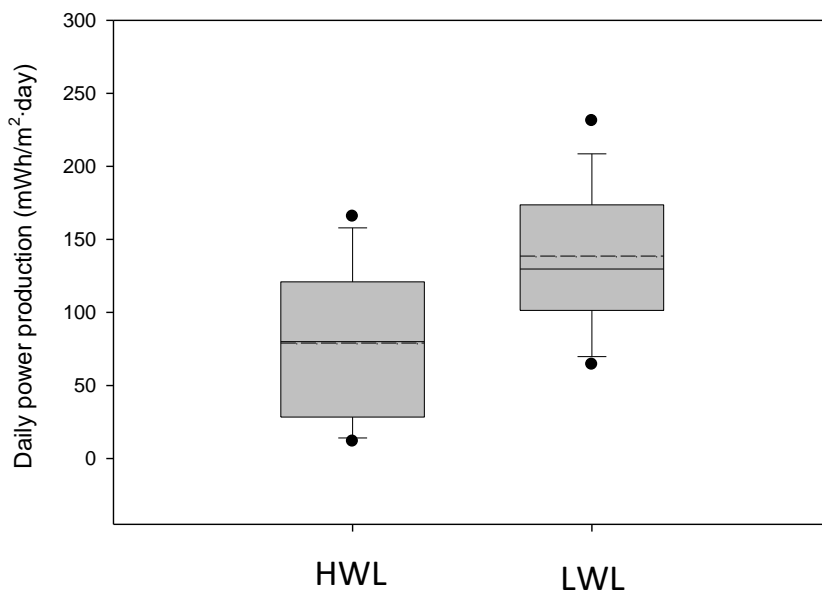
Figure 4.3.7 Daily energy production during high water level variation conditions (HWL) and low water level variation conditions (LWL)

4.3.3.5 *Contribution of macrophytes to water table variation*

As it has been discussed evapotranspiration had a great effect on CW-MFCs performance. The contribution of macrophytes on evapotranspiration and thus on water table variation was assessed. Also the contribution of macrophytes on electrical output generated by microbial fuel cells (MFCs) was quantified.

The effect of plants on MFC voltage patter is shown in Figure 4.3.8. From Figure 4.3.8 it can be seen that once the effect of plants was inhibited (by covering them), MFC water table variations drastically changed from a very marked daily fluctuation (when plants were not covered) to a more stable level when they were covered. Voltage generated followed the same pattern. A reduction of a -47% and -71% was detected in daily maximum current and power densities generated (Figure 4.3.9). As mentioned above, plants evapotranspiration in wetlands may cause significant water level variation and, thus, may influence oxygen availability at the cathode. To this extent, current results evidence that oxygen availability at the cathode under periods of low water level variation may increase MFC efficiency.

Even though it was demonstrated that there was an influence of macrophytes on the electrical output, this influence was actually a consequence of water table variations which, in turn, were a consequence of evapotranspiration. However, it is described that plants also supply oxygen and carbon based compounds through the roots. Therefore, the oxygen may have an influence on the redox potential within the wetland, affecting MFCs performance by increasing redox conditions at the cathode (positive effect) or at the anode (negative effect). At the same time, the easily biodegradable compounds given to the system, could be used by microbial communities in the anode to produce electricity from their oxidation (positive effect). By means of the experimental set-up described above, the influence of the oxygen and exhudates supplied by roots, could not be detected. It could be suggested that the very reduced environment fostered by the anaerobic reactor and also the high concentrations of organic matter inside the wetland, may have masked the effects of macrophytes and therefore, an appropriate design must be implemented to test this potential influence plants may have on MFCs performance.
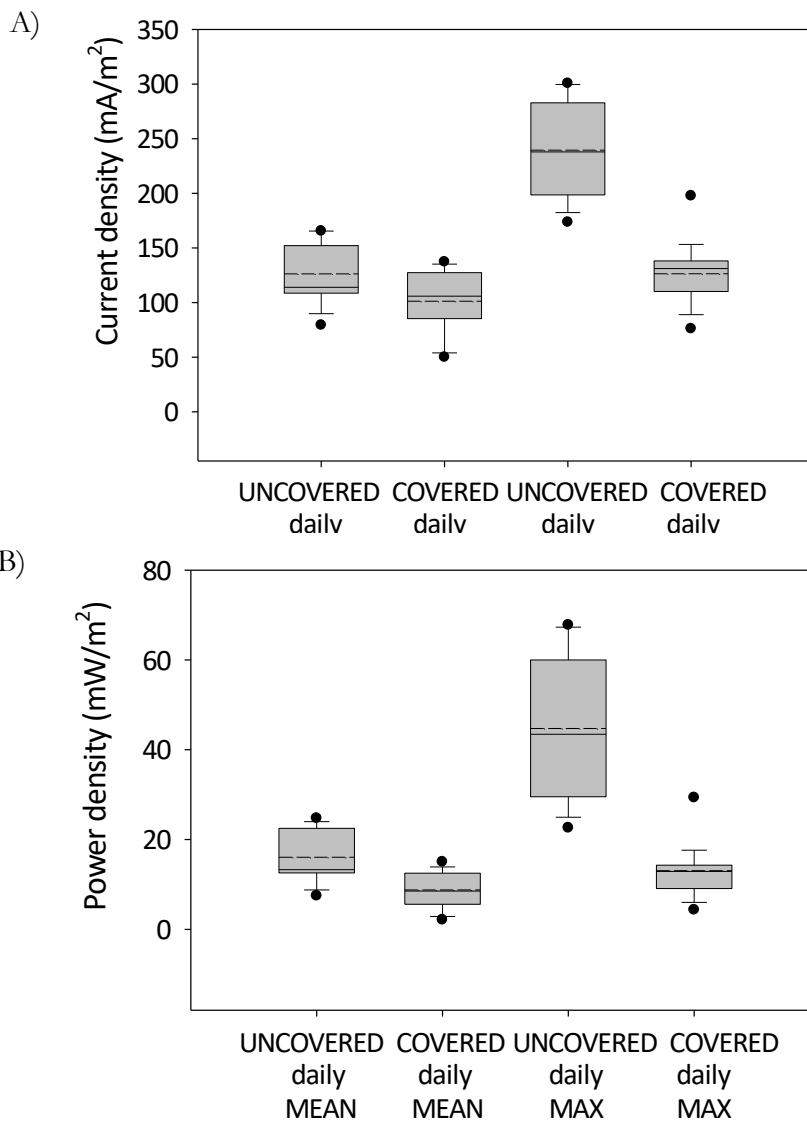
Figure 4.3.8 Influence of macrophytes coverage on current (A) and power densities (B)
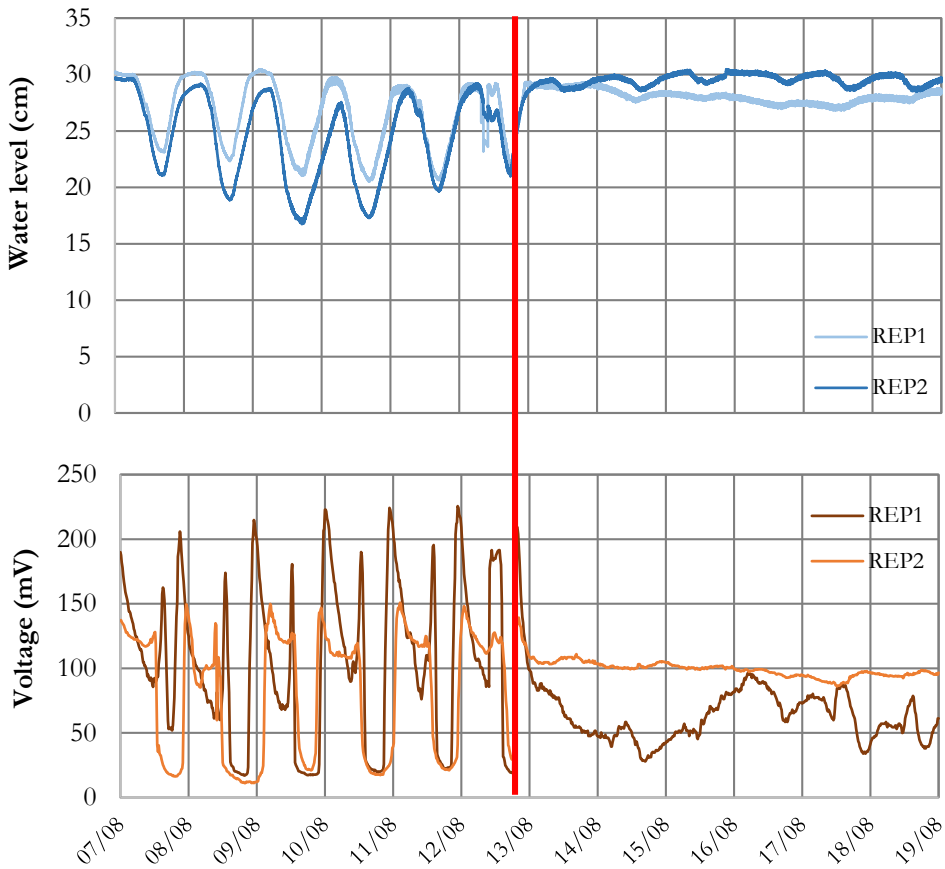
Figure 4.3.9 Influence of water level variation (top graph) caused by macrophytes evapotranspiration on MFC cell voltage (bottom graph). Red line indicates the moment when plants were covered

4.3.3.6 **Effect of electrodes distance on energy production in lab-scale MFCs**

As shown in Figure 4.3.10, voltage generated by the lab-scale CW-MFC decreased with the distance between electrodes. Accordingly, while at a distance of 10 cm, the average voltage was 455±19 mV, at a distance of 20 cm it dropped down to 103±20 mV. Actually, a linear relationship could be stablished between both parameters with a correlation factor (R-square value) of 0.96. These results are in accordance with bibliography in which it is widely established that the distance between electrodes is an influencing factor for conventional MFCs performance (Ghangrekar and Shinde, 2007). From the

results obtained in this lab-scale experiment, the distance between electrodes in the pilot plant was chosen to be 10 cm.
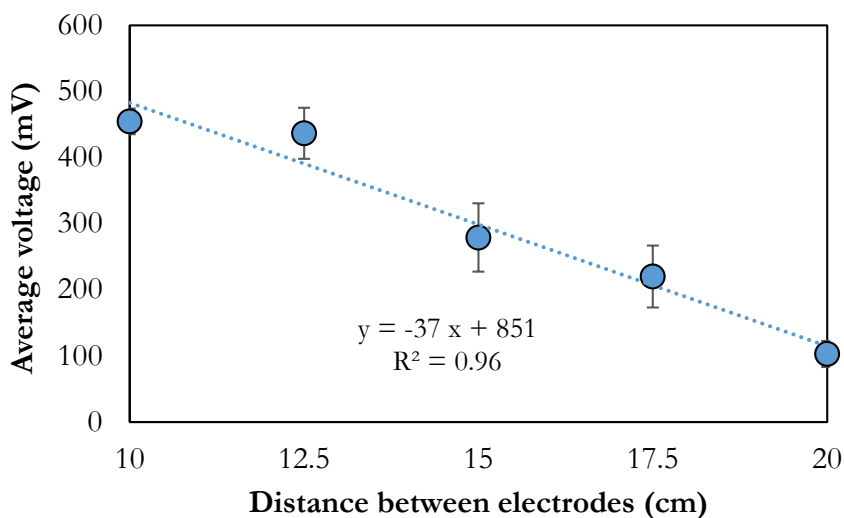
Figure 4.3.10 Influence of distance between electrodes on voltage produced by CW-MFCs

4.3.3.7 *Significance of energy production with MFC in the context of constructed wetlands*

Constructed wetlands (CWs) is a technology that consumes little energy for the treatment of domestic wastewater (<0.1 KWh/m^3) (Kadlec and Wallace, 2009). However, due to its large surface requirements (about 5 m^2/PE) CWs implementation is generally restricted to the sanitation of small human settlements (<10,000 inhabitants). In order to not only overcome the large surface requirements of CWs but also increase its treatment efficiency, active aeration has been considered a suitable strategy (Austin and Nivala, 2009; Kadlec and Wallace, 2009). However, CWs aeration increases the energy devoted to the treatment of wastewater when compared to passive, more traditional wetlands configuration. Current figures of energy consumption for completely aerated wetlands range from 0.16 to 0.49 kWh/m^3 (Austin and Nivala, 2009; Kadlec and Wallace, 2009). Under this condition we can

estimate that MFC implemented in CWs using the energy production figures here reported may cover less than 1% of the total energy demand (note that for the estimation we have assumed that one PE generates about 0.15 m^3/day of wastewater and that MFC energy production is that of 131 mWh/m^2.day and that only one third of the wetland is actually suitable for energy production via MFC since most of the COD is consumed within the first third of the wetland length). However, recent works carried out on the optimization of wetlands aeration have shown that the aeration of a small surface area of horizontal sub-surface flow constructed wetlands (8% of the total area) might be enough to increase treatment efficiency and reduce surface requirements (Labella et al., 2015). Under this situation the energy requirements are lower when compared to full surface aeration (0.029 $kWh/m^2 \cdot year$). Therefore, under these lower energy requirements for aeration the energy provided to the wetlands by means of MFCs would be about the 50% of the total energy demand (note that for the estimation we have assumed the same conditions as in the previous calculation).

4.3.3.8 *Influence of oxygen availability on the internal resistance: Impedance analysis*

As mentioned before, water level variation influenced the oxygen availability at the cathode, which in turn affected cells' performance. In this section this influence is assessed in terms of MFCs' overpotentials and ohmic losses. To this aim, two polarization curves were performed during the first period of MFC operation. One of the polarization curves was performed when cathode position was under sub-optimal conditions (cathode submerged 2 cm below water level) (Figure 4.3.11 A) whereas the other polarization curve was performed close to optimal conditions (cathode was in line with water level) (Figure 4.3.11 B). Overpotentials are generally current dependent and can occur both at the anode and the cathode. Logan et al. (2006) divides them into activation losses, bacterial metabolism losses and mass transport or concentration losses. The latter arise due to insufficient mass transfer of chemical species to or from the electrode which limits the reaction rate (Logan, 2008). Results shown in Figure 4.3.4 confirm that the lack of oxygen availability at the cathode when it was submerged increased concentration losses to the extent of preventing electrons to flow. Accordingly, Rabaey et al. (2008) reports that oxygen reduction at non-

catalysed materials cause large overpotentials. Moreover, open circuit voltage and internal resistance are also a manner to analyse MFCs' performance. The internal resistance, which is also current dependent, is defined as the sum of all internal losses (Logan et al., 2006) and it is reported that the cathode is one of the main factors contributing to it (Fan et al., 2008). In fact, the cathode as the power limiting factor has also been reported in a plant-MFC (Strik et al., 2008).

A)
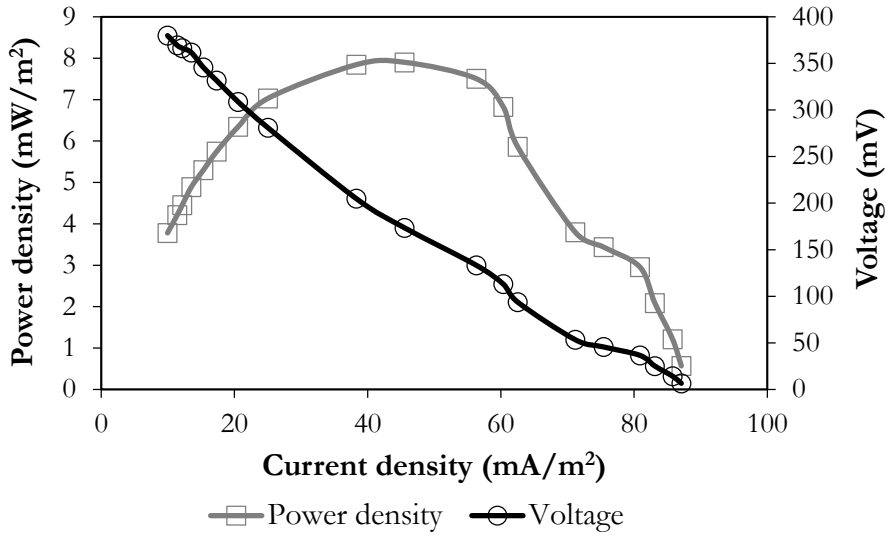
B)
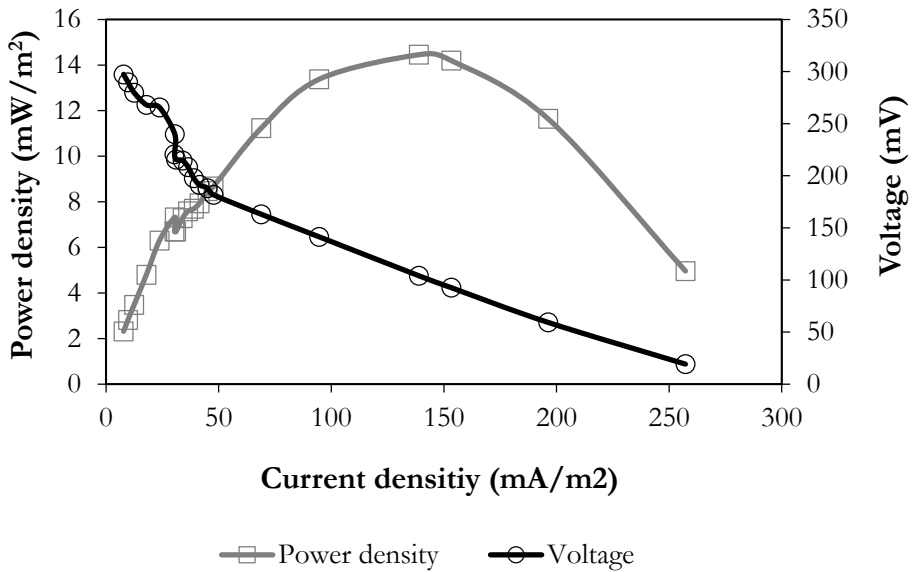
Figure 4.3.11 Polarization curves made on the same MFC (the same replicate) during normal operation: A) under optimal cathode position (in line with water level) and B) under suboptimal cathode position (2 cm below water level).

Polarization curves were made in order to electrochemically characterize MFCs working under two different cathode positions relative to water level. Maximum power and their corresponding current densities registered were 7.9 mW/m² and 45.5 mA/m² in the case of MFC under suboptimal cathode conditions (that is to say cathode submerged about 2 cm) and 14.5 mW/m² and 138.8 mA/m² in the case of MFC close to optimal conditions (cathode in line with water level). Accordingly, the internal resistances estimated from the polarization curves were 5080Ω and 1000Ω, respectively. This result is in accordance to the fact that the maximum power is achieved when the internal resistance is equal to the external (Lefebvre et al., 2011). Internal resistance can be estimated as the slope of the linear section of the polarization curve (Logan et al., 2006). Results obtained from these estimations showed that under both conditions the estimated internal resistance was in the range of external resistances at which maximum power was reached (6466Ω and 1064Ω for suboptimal and optimal cathode conditions respectively) and therefore that at maximum point $R_{ext}=R_{int}$. Overall, this analysis based on impedance shows that cathode position under optimal conditions reduces the internal resistance of the system and, therefore, maximises cell performance.

4.3.4 Conclusions

- Water level variation in constructed wetlands caused by evapotranspiration has a great effect on microbial fuel cell performance.
- Cathode shall remain between 1 to 2 cm above water level in order to optimize cell performance.
- Microbial fuel cell having a fixed cathode and operated under high water level variation produced about 40% less energy (80±56 mWh/m²·day) than under low water level variation (131±61 mWh/m²·day). Therefore, cell architecture shall address the possibility to cope with intense water level variation to optimise cell performance.
- Water level variation influences cathode performance by increasing the overall internal resistance of the system.

- Macrophytes had a clear impact on evapotranspiration and thus on water table variation. When macrophytes contribution was removed both voltages and water level fluctuation notably decreased.
- Voltage generated by the lab-scale CW-MFC decreased with the distance between electrodes.

Pure culture studies of the electricigen *Geobacter lovleyi* identify limitations of CW-MFCs

Abstract

Microbial Fuel Cells implemented in Constructed Wetlands (CW-MFCs) show limited performance although the anode electrodes are predominantly colonized by electricigens most closely related to *Geobacter lovleyi*. Thus, we investigated the parameters that limit the growth and electroactivity of the model representative *G. lovleyi* strain SZ. Acetate, but not formate or lactate, supported the reduction of fumarate and Fe(III) citrate but growth was limited by the high sensitivity of the bacterium to oxygen. Acetate also supported the growth and electroactivity of anode biofilms under highly reducing conditions and optimal anode potentials (450 mV versus standard hydrogen electrode). Yet, even under optimal conditions, electrode coverage was poor consistent with the lack of key *c*-type cytochromes required for efficient electrode reduction by anode biofilms. The results suggest that the low electrochemical performance of CW-MFCs is the result, at least partially, of the low oxygen tolerance of environmental *G. lovleyi* strains and their inability to efficiently colonize and form electroactive biofilms on the electrodes while oxidizing the range of electron donors available in constructed wetlands. The implications of these findings for the optimization of CW-MFCs are discussed.

Constructed wetland MFCs → Mixed-species anode biofilms → Pure culture MECs
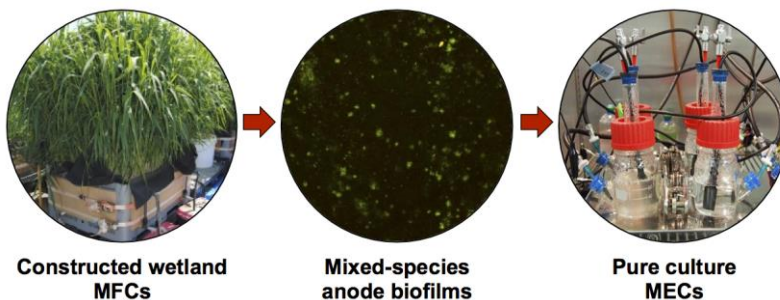
4.4.1 Introduction

Horizontal subsurface flow constructed wetlands (HSSF CW) are natural wastewater treatment systems where organic matter is oxidized by means of physical, chemical and biological processes under mainly anaerobic conditions (Baptista et al., 2003). During treatment, a redox gradient is naturally established between the top layer exposed to air and the deeper anaerobic areas of the treatment bed that can be exploited to harvest an electrical current with a sediment microbial fuel cell (MFC) (Corbella et al., 2014). Although MFCs operating in HSSF CW (CW-MFCs) increase organic matter removal efficiency, power density and coulombic efficiencies are generally low (below 50 mW/m^2 and 4%, respectively) (Doherty et al., 2015). This suggests that the anode biofilms in CW-MFCs may limit their performance, as observed in other electrochemical systems that process domestic wastewater (Logan and Rabaey, 2013). The efficient processing of wastewater by anode biofilms depends on the syntrophic interactions among the biofilm members, which first hydrolyze the complex substrates and then ferment them to generate electron donors (e.g., acetate, formate, and lactate) for the electrogenic population (Cusick et al., 2010; Kiely et al., 2011). Parameters such as substrate type, temperature, redox potential, and pH fluctuate widely in HSSF CW and affect the efficiency of the syntrophic communities that drive CW-MFCs. For example, changes in the chemical composition of the wastewater influence substrate availability and the type and relative abundance of bacteria that grow in the anode biofilms (Corbella et al., 2015a). Further, several pathways for organic matter degradation can be established in HSSF CW, each providing a different range of electron donors to support the growth and activity of the electricigenic population (Faulwetter et al., 2009). Acetate, a preferred electron donor for efficient electricigens in the genus *Geobacter*, (Speers and Reguera, 2012) is the most common electron donor generated in these anaerobic degradation pathways. Consistent with this, bioelectrochemical systems fed with domestic wastewater often enrich for *Geobacter* electricigens in anode biofilms (Corbella et al., 2015a; Cusick et al., 2010; Fang et al., 2013). More than half of the 16S rRNA sequences retrieved from anode biofilms grown in a single chamber microbial electrolysis cell (MEC) fed with domestic wastewater were, for example, most closely related to electricigenic species of *Geobacter* available in pure culture (*Geobacter*

metallireducens, *Geobacter sulfurreducens*, *Geobacter lovleyi* and *Geobacter uraniireducens*) (Cusick et al., 2010). Similarly, operational Taxonomic Units (OTUs) most closely related to members of the Geobacteraceae family were prominent members in the anode biofilms of an active CW-MFC fed with the effluent of a hydrolytic upflow sludge blanket reactor, with the highest relative abundance (13-16% of the total OTUs) corresponding to *G. lovleyi* (Corbella et al., 2015a). The enrichment of *Geobacter*-like sequences in anodic biofilms of active CW-MFCs is consistent with the availability of the acetate in constructed wetlands (Bagley et al., 2000). More reduced compounds such as lactate and formate are also produced (Bagley et al., 2000), but they are not always efficiently assimilated by model *Geobacter* electricigens (Speers and Reguera, 2012).

The relative abundance of *G. lovleyi* OTUs in active CW-MFCs is influenced by the chemical composition of the wastewater, (Corbella et al., 2015a) suggesting that substrate availability influences the composition of the syntrophic community and the type of electron donors available for the growth of this type of electricigens (Corbella et al., 2015a). This suggests that further optimization of CW-MFCs must consider the factors that limit the growth and electroactivity of *G. lovleyi* on the anode electrodes. In contrast with the amount of information that is available for other model *Geobacter* representatives such as *G. sulfurreducens* and *G. metallireducens* (reviewed in (Lovley et al., 2011)), little is known about the physiological constraints that may limit the growth and electrochemical activity of *G. lovleyi*. The first strain of *G. lovleyi* recovered in pure culture, strain SZ, was isolated from non-contaminated creek sediment microcosms based on its ability to grow by coupling the oxidation of acetate to the reductive dechlorination of tetrachloroethene (PCE) to cis-1,2-dichloroethene (cis-DCE) (Sung et al., 2006). Closely related 16S rRNA gene sequences have been retrieved from environments where dechlorination is an active process, placing *G. lovleyi*-like sequences in a distinct, dechlorinating clade within the metal-reducing *Geobacter* group, which model electricigens belong to (Amos et al., 2007; Sung et al., 2006). Strain Z is also capable of direct nitrification to ammonium and retains the ability to reduce Fe(III), the major hallmark of the physiology of *Geobacter* electricigens (Sung et al., 2006). However, its genome shows marked reductions in the number of c-type cytochrome genes required for metal reduction (Wagner et al., 2012). Furthermore, anode biofilms grown with

acetate and fumarate produced electricity when the electrode was poised at a metabolically oxidizing potential, though MEC performance was low compared to the model electricigen *G. sulfurreducens* (Strycharz et al., 2008). In addition, strain SZ was able to use the MEC's cathode as sole electron donor while coupling the reduction of fumarate and PCE at rates comparable to those of in cultures with acetate donor (Strycharz et al., 2008). Growth with a cathode may have involved the formation of cathodic H_2, which strain SZ can use as electron donor as well (Sung et al., 2006).

In this study, we used *G. lovleyi* strain SZ as a laboratory model representative to gain insights into the constraints that limit the performance of electricigens in the anode biofilms of CW-MFCs. Using the well-known metabolism of the model electricigen *G. sulfurreducens* as a reference (Speers and Reguera, 2012), we optimized the culture conditions that support the growth of strain SZ with acetate and soluble electron acceptors such as fumarate and Fe(III) and tested the ability of this organism to use two other organic acids (lactate and formate) commonly found in CW. Lastly, we investigated the growth and electrochemically activity of anode biofilms in MECs and describe the metabolic and physiological constraints that limit current production by *G. lovleyi* and their relevance to the optimization of CW-MFCs.

4.4.2 Materials and methods

4.4.2.1 Bacterial strains and culture conditions

G. lovleyi SZ, kindly provided by Dr. Dawn Holmes (Western New England University), was used throughout the study. The strain was routinely cultured anaerobically in DB medium (Speers and Reguera, 2012) supplemented with 2.5 mM cysteine HCl as a reducing agent and with sodium acetate (20 mM) and sodium fumarate (40 mM) as the electron donor and acceptor, respectively (DBAF medium). The growth medium was dispensed into tubes (10 ml) and serum bottles (50 ml) and sparged with N_2:CO_2 (80:20) (Speers and Reguera 2015) before sealing the vessels with rubber stoppers and autoclaving for 30 minutes.

When indicated, acetate, lactate and formate were provided to the medium at concentrations (9 mM sodium acetate, 6 mM D,L-lactate, or 36 mM sodium formate) that provided equimolar amounts of electrons from the electron donors. Growth with each of these electron donors was studied using fumarate (40 mM) or Fe(III) citrate (80 mM) as electron acceptor. For these experiments, cells were first grown to late exponential phase (~ 0.4 OD_{600}) in DB medium with the electron donor-acceptor pair to be tested, harvested by centrifugation (3,200 x g, 10 min, 30°C), washed once in sterile medium, and resuspended in 0.5 ml of the growth medium to prevent nutrient carry-over. When indicated, the centrifugation and washing steps were omitted and the cultures were transferred three times in the same medium before calculating the growth rates for each electron donor-acceptor pair. All incubations were at 30ºC. Growth in fumarate cultures was monitored spectrophotometrically (OD_{600}) and in Fe(III) citrate cultures, as the amount of HCl-extractable Fe(II) resulting from the reduction of Fe(III) (Lovley and Phillips, 1986) measured using the ferrozine assay (Stookey, 1970).

4.4.2.2 Analytical techniques

Organic acids in filtered (0.45 μm syringe filters, Thermo Scientific Titan3) culture supernatant fluids were identified and quantified by high-performance liquid chromatography (HPLC) (Waters, Milford, MA) as reported elsewhere (Speers and Reguera, 2012). Cysteine was measured spectrophotometrically (OD_{412}) as free thiols with the Ellman's Reagents, as described previously (Robyt and White 1987), and in reference to L-cysteine hydrochloride standards. When indicated, the total cell protein content in the culture was estimated using Pierce BCA Protein Assay Kit (Thermo Scientific) using a previously published method (Young et al., 2012).

4.4.2.3 MECs and confocal microscopy

Microbial Electrolysis Cells (MECs) were the same dual-chambered, H-type fuel cells described previously (Speers and Reguera, 2012). Anode and cathode chambers were separated by means of a Nafion membrane (N117, Ion Power, Inc. New Castle, DE) and each chamber contained 90 ml of DB-acetate (1 mM) medium and a graphite rod electrode (1.27 cm diameter, 99% metal basis, 12 cm^2 anode surface area). The anode and cathode chambers

were sparged with $N_2:CO_2$ (80:20) to ensure anaerobiosis and the anode potential was set with a potentiostat (VSP, BioLogic, Claix, France) at -0.179V, 0.240V or 0.561V versus a 3M Ag/AgCl reference electrode (Bioanalytical Systems, Inc., West Lafayette, IN). The anode chamber was then inoculated with cells of *G. lovleyi* and, when indicated, of *G. sulfurreducens* harvested by centrifugation (3,200 rpm, 10 min, 30°C) from stationary phase cultures grown with DBAF medium and suspended in 10 ml of DB medium with acetate. All experimental conditions were tested at least in duplicate MECs. Culture broth samples were periodically removed from the anode chamber and analyzed by HPLC. When indicated, anode biofilms were grown to the point of maximum current, stained with the BacLight™ viability kit (Molecular Probes), and examined by Confocal Laser Scanning Microscopy (CLSM) using a FluoView FV1000 inverted microscope system (Olympus, Center Valley, PA) as described elsewhere (Speers and Reguera, 2012).

4.4.3 Results and discussion

4.4.3.1 *Optimum redox potential for growth with acetate during the reduction of fumarate of Fe(III) citrate*

Because oxygen intrusions are common in CW, which could inhibit the growth and electroactivity of electricigens, we investigated the influence of the medium redox potential in the growth of *G. lovleyi* strain SZ. Although the media preparation involved extensive sparging of the broth and headspace with oxygen-free gases ($N_2:CO_2$) (Speers and Reguera 2015), growth was only observed in cultures supplemented with cysteine as a reducing agent (Figure 4.4.1). Strain SZ coupled, for example, the oxidation of acetate (20 mM acetate or 160 mM electron equivalents) to the reduction of Fe(III) citrate (Figure 4.4.1 A) but only when the cultures were supplemented with cysteine (2.5 mM). Replacing the cysteine with a mild reducing agent such as $FeCl_2$ (2.5 mM) (Straub et al. 2005) did not support Fe(III) reduction (data not shown). Generation times in the cultures with cysteine were 18 ± 1 h (average and standard deviation of triplicate cultures) and lower (8 ± 0.1 h) after three direct transfers in the same medium to avoid the stress caused to the cells during the centrifugation and washing steps. These doubling times are within the ranges (~ 10 h) reported for *G. sulfurreducens* grown in the same DBA-Fe(III) citrate medium but without cysteine (Speers and Reguera, 2012). Furthermore, the

total amount of Fe(II) (~ 50 mM) reduced by *G. lovleyi* was as in the *G. sulfurreducens* cultures (Speers and Reguera, 2012), indicating that once the medium was pre-reduced, strain SZ was able to couple the oxidation of acetate and Fe(III) as efficiently as *G. sulfurreducens*.
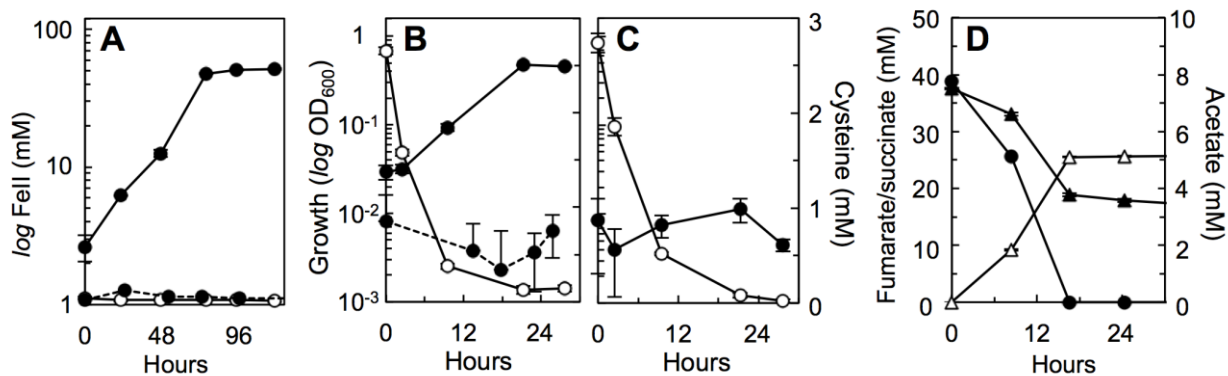
Figure 4.4.1 Effect of cysteine on growth of *G. lovleyi* with acetate and Fe(III) citrate (A) or fumarate (B-D). A. Growth (log of the acid-extractable Fe[II]) with 15 mM acetate and 80 mM Fe(III) citrate in the presence (solid symbols) or absence (open symbols) of 2.5 mM cysteine. An uninoculated control with cysteine is shown as well (dashed line). B-C Growth (OD_{600}, solid symbols) and cysteine concentration (mM, open symbols) in DBAF cultures with (solid line) or without (dashed line) 2.5 mM cysteine (B) in reference to uninoculated controls (C). D. Oxidation of acetate (solid circles) coupled to the reduction of fumarate (solid triangles) to succinate (open triangles) in the DBAF cultures supplemented with 2.5 mM cysteine shown in B. All of the data points in panels A-D are average and standard deviation of triplicate samples

Addition of cysteine as a reducing agent also supported growth in cultures with acetate and fumarate (Figure 4.4.1 B). We tested various concentrations of cysteine ranging from 0.1 to 10 mM and we consistently measured maxima growth yields and shortest generation times at cysteine concentrations between 2 and 3 mM. Cultures supplemented with 2.5 mM (Figure 4.4.1 B) yielded, for example, a maxima cell biomass (measured as total cell protein at the point of maximum growth, when all the acetate was depleted) similar to that measured in the DBA-Fe(III) citrate cultures (~ 10 g protein per mol acetate consumed). Cysteine in these cultures was rapidly oxidized within the first 10 h of incubation (Figure 4.4.1 B) at rates comparable to the oxidation of cysteine in uninoculated controls (Figure 4.4.1 C), consistent with its abiotic oxidization to cysteine (Kaden et al., 2002). Exponential growth started after a short (2-3 h) *lag* phase, when approximately 1 mM of cysteine had been oxidized, and proceeded with doubling times of 6 ± 0.4 h (average and standard deviation of triplicate cultures) (Figure 4.4.1 B). And, as observed with the Fe(III)-citrate cultures, the *lag* phase was eliminated and doubling times were reduced (4.4 ± 0.1 h) when exponential phase cultures were sequentially transferred three times in the same medium with cysteine to avoid the cell centrifugation and washing steps prior to inoculation. These fast generation times are within the ranges we calculated (~ 4.6 ± 0.2 h) for the model electricigen *G. sulfurreducens* growing in the same DBAF medium but without cysteine (Speers and Reguera, 2012). Thus, the rates of acetate oxidation coupled to fumarate reduction are also comparable in the two strains, but growth by *G. lovleyi* requires the presence of sufficient concentrations of cysteine as a reducing agent. The absolute requirement to pre-reduce the medium sufficiently in order to support cell growth is in accordance with genomic data, which indicates that *G. lovleyi* cannot respire oxygen and lacks several key genes involved in oxygen tolerance and detoxification of reactive oxygen species (Wagner et al., 2012). Indeed, field experiments show significant (up to 50%) decreases in the relative abundance of *G. lovleyi* like sequences in uranium-contaminated sediments following the intrusion of oxygenated groundwater (Thomas et al., 2010). By contrast, *G. sulfurreducens* tolerates exposure to atmospheric oxygen for up to 24 h and can use oxygen as terminal electron acceptor for respiration under microaerophilic conditions, a metabolic capability that allows them to continue to grow in environments subjected to oxygen intrusions (Lin et al., 2004).

Although *G. lovleyi* was able to double every 4-5 hours, like *G. sulfureducens*, in acetate-fumarate cultures with cysteine, growth yields were significantly lower (OD_{600} maxima ~ 0.5 for *G. lovleyi*; Figure 4.4.1 B) than those reported (Speers and Reguera, 2012) for *G. sulfurreducens* (OD_{600} maxima, ~ 0.8). The high growth yields of *G. sulfurreducens* in cultures with acetate and fumarate have been attributed to the ability of this organism to assimilate some of the fumarate carbon, which diverts more of the acetate substrate (72.5%) for energy generation via respiration (Yang et al., 2010). To investigate a similar metabolic capability in *G. lovleyi*, we monitored acetate and fumarate consumption and the production of succinate (from the reduction of fumarate) or malate (from the assimilation of fumarate carbon) in *G. lovleyi* DBAF cultures that contained growth-limiting concentrations of acetate (~9 mM or 72 mM electron equivalents) (Figure 4.4.1 D). Acetate was consumed during the first 16 h and, concomitantly, fumarate was reduced to succinate. We estimated that approximately 19 (± 0.3) mM fumarate was consumed, or the equivalent of 60% of the electrons provided as acetate in the medium, suggesting that *G. lovleyi* diverted more acetate carbon (~ 40%) for biomass synthesis than *G. sulfurreducens* (27.5%) (Yang et al., 2010). Although the assimilation of acetate carbon in the TCA cycle generates malate (Segura et al., 2008), we did not detect any malate in the acetate-fumarate cultures of *G. lovleyi* but measured an excess succinate (~ 7 mM) that could not be accounted for by the fumarate that was reduced (Figure 4.4.1 D). As strain SZ can reduce malate to succinate (Sung et al., 2006), the excess succinate detected in the cultures likely resulted from the reduction of acetate-derived malate.

4.4.3.2 *Alternative electron donors supporting the growth of G. lovleyi*

The finding that *G. lovleyi* diverts more acetate carbon for assimilation than *G. sulfurreducens* suggests that metabolic differences do exist between *G. loveyi* and model electricigens that could limit the performance of CW-MFCs despite the availability of acetate as an electron donor. Organic acids such as lactate and formate are also available as electron donors in CW (Bagley et al., 2000). Thus, we investigated the ability of *G. lovleyi* to grow with these two organic acids (Figure 4.4.2) using the cultivation conditions with cysteine that supported optimal growth of *G. lovleyi* with acetate (Figure 4.4.1). Although formate and lactate are more reduced than acetate and can, therefore, theoretically produce higher cell voltages (-0.403 and -0.325 V versus standard

hydrogen electrode [SHE], respectively), and more energy for growth, than acetate (-0.277 V versus SHE) (Kiely et al., 2010), neither electron donor supported the growth of *G. lovleyi* in cultures with fumarate or Fe(III) citrate serving as the electron acceptor and supplemented with 2.5 mM cysteine (Figure 4.4.2 A and B, respectively). Moreover, fumarate concentrations in the cultures with formate and lactate as electron donors remained relatively constant throughout the incubation period and the reduced product of the reaction, succinate, did not accumulate in the culture broth (Figure 4.4.2 C). Similarly, electron donor consumption was only detected in the positive controls that contained acetate as the electron donor (Figure 4.4.2 C and D).
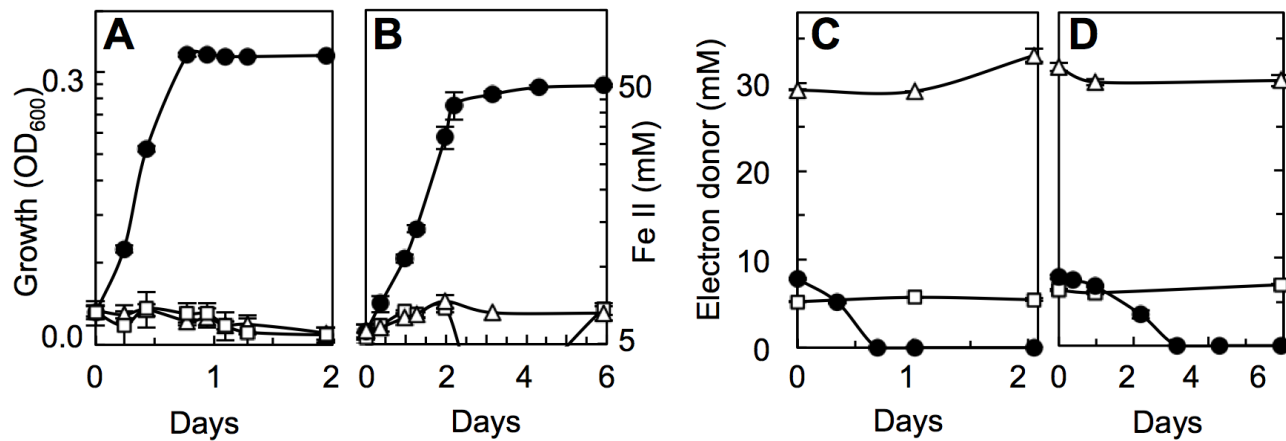
Figure 4.4.2 Growth (A-B) and electron donor consumption (C-D) of *G. lovleyi* with fumarate (A and C) or Fe(III) citrate (B and D) as electron acceptors, respectively. The electron donors tested were acetate (solid circles), lactate (open squares), and formate (open triangles). Other organic acids such as fumarate, succinate, malate, and pyruvate were also monitored in the culture broths (C and D) but were not detected

As formate and lactate can be oxidized by anode biofilms of the model electricigen *G. sulfurreducens* (Speers and Reguera, 2012), we used the BLAST engine (http://blast.ncbi.nlm.nih.gov/) to perform a comparative search of genes encoding key proteins needed for formate and lactate metabolism in *G. lovleyi*. As shown in Figure 4.4.3, *G. sulfurreducens* oxidizes formate to carbon dioxide in a reaction catalyzed by the formate dehydrogenase (FDH) enzyme and assimilates formate carbon with acetyl-CoA in a separate reaction catalyzed by the pyruvate formate lyase (PFL) enzyme (Speers and Reguera, 2012). The genome of strain SZ contains two FDH homologues (Glov_1164 and Glov_0899) (Wagner et al., 2012). However, their function in formate oxidation is unclear based on earlier studies (Sung et al., 2006) that showed that formate cannot be used as a source of electrons under PCE- or Fe(III)-reducing conditions. Similarly, formate does not sustain the coupled oxidation of H_2 and reduction of PCE and Fe(III), suggesting that formate cannot be assimilated for carbon either (Sung et al., 2006). Indeed, we were unable to identify PFL-like sequences in the genome of *G. lovleyi* nor was growth promoted in formate cultures supplemented with 0.1 mM acetate to provide the acetyl-CoA substrate for formate carbon assimilation (data not shown). This contrasts with the growth-promoting effect that such low levels of acetate supplementation have in formate assimilation in *G. sulfurreducens* (Speers and Reguera, 2012). Thus, even with active FDH enzymes for formate oxidation, the lack of a PFL enzyme would prevent the cells from assimilating formate carbon to generate pyruvate for gluconeogenesis, biomass synthesis, and other assimilatory reactions (Figure 4.4.3).
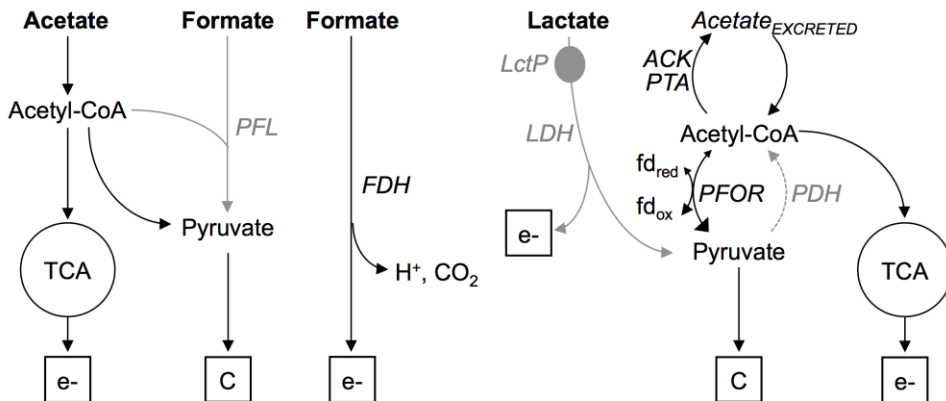
Figure 4.4.3 Metabolic pathways used by *G. sulfurreducens* for the oxidation (e⁻) and carbon assimilation (C) of acetate, formate, and lactate substrates (in bold) and identification of reactions (in gray) absent in *G. lovleyi*. Dashed arrows indicate reactions that operate in cultures with fumarate. Enzyme abbreviations, from left to right: PFL, pyruvate formate lyase; FDH, formate dehydrogenase; LctP, lactate permease; LDH, lactate dehydrogenase; ACK, acetate kinase; PTA, phosphotransacetylase; PFOR, pyruvate ferredoxin oxidoreductase; PDH, pyruvate dehydrogenase

As with the formate cultures, acetate additions to lactate cultures of *G. lovleyi* (Figure 4.4.2) did not support growth with either fumarate or Fe(III) citrate (data not shown), indicating that lactate cannot be used as electron donor during the reduction of fumarate and Fe(III), as also reported for PCE reduction (Sung et al., 2006). Earlier studies did indicate, however, that lactate, like acetate, can be assimilated for carbon to sustain the reduction of PCE and Fe(III) with H_2 as electron donor (Sung et al., 2006). However, a comparative search in the genome of *G. lovleyi* failed to identify any lactate permeases, including homologs of the two lactate transporters annotated in the genome of *G. sulfurreducens* (GSU1622 and GSU0226). Furthermore, the SZ genome does not contain any genes annotated as lactate dehydrogenases (LDH), which catalyze the partial oxidation of lactate to pyruvate (Figure 4.4.3). Similarly, our search retrieved no significant matches for homologs of the two subunits of the glycolate oxidase (GO) enzyme of *G. sulfurreducens* (GSU1623 and GSU1624), which is structurally homologous to LDH but has a reduced LDH activity (Speers and Reguera, 2012).

Also unclear is how any lactate-derived pyruvate could be fully oxidized in the TCA cycle by *G. lovelyi*. Its genome does not encode homologs of the subunit A of the pyruvate dehydrogenase E1 complex (PDH), which provides a route to convert pyruvate into acetyl-CoA substrate for the oxidative TCA cycle (Figure 4.4.3). The genome of *G. lovleyi* does contain five gene clusters (one on plasmid pSZ77) encoding pyruvate ferredoxin/flavodoxin oxidoreductase (PFOR) complexes (Wagner et al., 2012), which could catalyze the conversion of pyruvate into acetyl-CoA (Figure 4.4.3) These PFOR complexes are homologous to the PFOR enzyme of *G. sulfurreducens* that has been proposed to preferentially work in the opposite direction to promote the flux of acetyl-CoA to gluconeogenetic pyruvate (Yang et al., 2010). The preferential direction of the PFOR reactions towards pyruvate for gluconeogenesis in *G. lovleyi* is supported by the greater amounts of acetate carbon that we estimated to be assimilated by strain SZ (40%) compared to *G. sulfurreducens* (27.5%). Yet strain SZ does use pyruvate as sole electron donor (Sung et al., 2006), suggesting that one, if not more, of the PFOR enzymes may preferentially operate in the opposite direction to divert lactate-derived pyruvate to acetyl-CoA for its full oxidation in the TCA cycle (Figure 4.4.3).

The genome of *G. lovleyi* also contains two genes (Glov_1754 and Glov_1210) annotated as acetate kinase (ACK) and phosphotransacetylase (PTA), respectively. *G. sulfurreducens* diverts pyruvate as acetyl-CoA substrate for the ACK/PTA pathway to excrete excess carbon as acetate in an ATP-yielding reaction (Figure 4.4.3) (Speers and Reguera, 2012). This suggests that *G. lovleyi* could also partially oxidize lactate to acetate to balance excess fluxes of carbon while generating energy for growth. However, we did not measure any acetate in cultures with lactate (Figure 4.4.2 C and D). Thus, other rate-limiting steps such as inefficient lactate transport or oxidation to pyruvate likely prevented the oxidation and assimilation of lactate and prevented growth of *G. lovleyi* with lactate.

4.4.3.3 Growth and electrochemical activity of G. lovleyi anode biofilms in microbial electrolysis cells (MECs)

We also investigated the growth and electrochemical activity of strain SZ in MECs fed with acetate (Figure 4.4.4). Earlier studies in MECs

(Strycharz et al., 2008) demonstrated that anode biofilms pre-grown with acetate and fumarate could couple the oxidation of acetate to the reduction of an anode electrode poised at 500 mV (vs SHE) once fumarate was removed from the medium. Yet current by the anode biofilms was only initiated after a considerable *lag* phase (around 5 days) and maximum current was 10-times lower than that recorded in MECs driven by *G. sulfurreducens* and operated under similar conditions (Strycharz et al., 2008). Furthermore, the anode biofilms were thinner (~12 µm) than those formed by *G. sulfurreducens* (~ 50 µm) in similar systems (Strycharz et al., 2008). As earlier studies did not investigate if *G. lovleyi* could couple the growth of anode biofilms to the reduction of the anode electrode, a critical step in establishing electrochemically active biofilms on the anodes of CW-MFCs, we grew *G. lovleyi* in MECs fed an initial concentration of 1 mM acetate. For these experiments, the anode electrode was poised at a potential (450 mV vs SHE) that is optimal for the growth of anode biofilms of the model electricigen *G. sulfurreducens* (Speers and Reguera, 2012). As shown in Figure 4.4.4 A, current production started soon after inoculation but required pre-reduction of the medium with 2.5 mM cysteine to provide optimal redox potential levels for growth. Under these optimal conditions, current production increased over the course of ten days until it reached maximum current (0.086 ± 0.004 mA, average and standard error of duplicate MECs), which was sustained for several more days until all of the acetate was depleted (Figure 4.4.4 A). By contrast, control MECs driven by *G. sulfurreducens* grown under the same conditions with cysteine reached a maximum current of 0.642 mA in less than 2 days. Moreover, coulombic efficiencies (CE) in MECs driven by *G. lovleyi* ranged from 30 to 40%, which is less than half of those estimated for *G. sulfurreducens* (~ 80%) (Speers and Reguera, 2012).
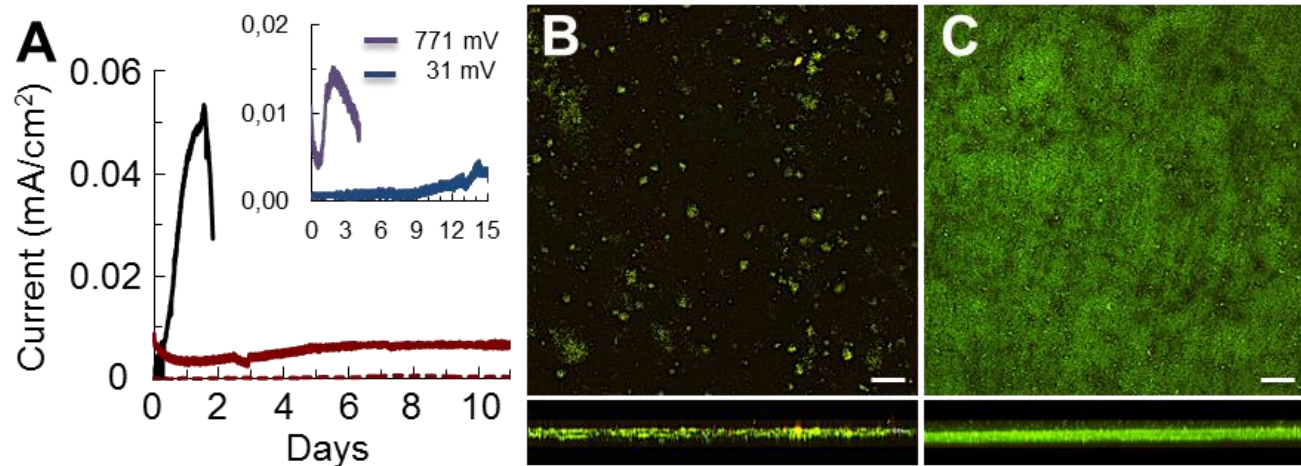
Figure 4.4.4 Current density (A) and growth of anode biofilms (B-C) in MECs driven by *G. lovleyi* or *G. sulfurreducens* fed with an initial concentration of 1 mM acetate. A. Current density (mA/cm²) by *G. lovleyi* (maroon) and *G. sulfurreducens* (black) in MECs poised at an anode potential of 450 mV (vs. SHE) with (solid line) or without (dashed line) 2.5 mM cysteine. Inset, current density by *G. lovleyi* in MECs with cysteine as a function of the anode potential (771 or 31 mV vs. SHE). Axis units are as in panel A. B-C. Top and side views of CSLM projections of anode biofilms of *G. lovleyi* (B) and *G. sulfurreducens* (C) collected at the point of maximum current from MECs at 450 mV (vs. SHE) and supplemented with cysteine (A). The biofilms were stained with the BacLight™ viability dies (green, live; red, dead). Scale bar, 20 μm.

Even when operated under optimal conditions, the growth and electrochemical activity of *G. lovleyi* was limited by poor electrode colonization and biofilm growth. Indeed, Confocal Laser Scanning Microscopy (CLSM) of anode biofilms from MECs with cysteine and with anodes poised at the optimal potential (450 mV vs. SHE) revealed poor electrode coverage by *G. lovleyi* cells, in contrast to the saturating biofilms formed by *G. sulfurreducens* (Figure 4.4.4 B and C, respectively). Though sparse, the anode microcolonies formed by strain SZ reached average thickness (9.1 ± 1.2 μm) similar to that of the saturating biofilm of *G. sulfurreducens* (10.8 ± 2.3 μm). To reach this thickness, *G. sulfurreducens* couple growth on the anode electrode to electron transfer to the underlying electrode, a process that requires the combined redox activities of matrix-associated *c*-type cytochromes such as OmcZ$_S$ and conductive pili (Steidl et al., 2016). The genome of *G. lovleyi* contains a protein homologous to the peptide subunit or pilin that polymerizes to make the conductive pili of *G. sulfurreducens* (Wagner et al., 2012). However, we were unable to identify a clear homolog of OmcZ (GSU2078), the precursor of the matrix-associated *c*-type cytochrome OmcZ$_S$ (Inoue et al., 2010) that concentrates near and on the electrode and is required for efficient electron transport to the anode surface (Inoue et al., 2011). Outer membrane cytochromes required for extracellular electron transfer to solid-phase electron acceptors such as OmcS (Mehta et al., 2005) did not retrieve a clear homolog either. Also absent in the genome of strain SZ are *c*-type cytochromes with more than 12 hemes (Wagner et al., 2012), which have been proposed to store electrons and continue energy generation through a proton motive force until the cell establishes electronic contact with the electron acceptor (Esteve-Núñez et al., 2008). Indeed, the genome of *G. lovleyi* contains less cytochrome-encoding genes than any other sequenced *Geobacter* genome (Wagner et al., 2012). Thus, the inability of *G. lovleyi* to colonize and respire the electrode efficiently could result, at least partially, from the lack of *c*-type cytochromes needed for the cells to establish electronic contact with the electrode.

Poising the anode potential at 31 mV (vs. SHE) to mimic the theoretically lower energy gain derived from the two-electron reduction of fumarate to succinate reduced MEC performance proportionally (Figure 4.4.4 A, inset). In *G. sulfurreducens,* the ability of the cells to reduce low potential,

solid-phase electron acceptors depends on the expression of the inner membrane cytochrome CbcL (Zacharoff et al., 2016). A search of the genome of *G. lovleyi* retrieved no clear homologs of CbcL, consistent with the inability of the anode biofilms to efficiently reduce the electrode when poised at low metabolically oxidizable potentials. We did identify a gene (Glov_2063) encoding a protein homologous (63.9% identity and 72.8% similarity) to ImcH, an inner membrane cytochrome of *G. sulfurreducens* that is required for extracellular electron transfer at high (> 240 mV versus SHE) redox potentials (Levar et al., 2014). We also set up MECs with anodes poised at the potential (771 mV versus SHE) of the Fe(III)/Fe(II) pair and observed some modest increases in maximum current (from 0.09 ± 0.01 to 0.21 ± 0.05 mA) and in the rates of current production (from 0.010 ± 0.003 to 0.190 ± 0.071 mA/day) in triplicate MECs (Figure 4.4.4 A, inset). However, confocal micrographs of anode electrodes from MECs operated at high potential showed very sparse colonization by individual cells, rather than a biofilm. Furthermore, acetate was not consumed in these MECs, suggesting that current production in these MECs may have been abiotic and involved cysteine, as previously reported for MFCs (Logan et al., 2005).

4.4.3.4 Implications for CW-MFC performance

The results presented herein demonstrate that *G. lovleyi* has metabolic and physiological constraints not reported for other model electricigens that are likely to limit the performance of CW-MFCs enriched with *G. lovleyi*-like sequences. Of special significance is the low oxygen tolerance of *G. lovleyi*, which requires the presence of oxygen scavengers to maintain the redox potential of the medium at a level sufficiently low to permit growth. This sensitivity contrasts with the oxygen tolerance and respiratory capacity of model electricigens such as *G. sulfurreducens*, a metabolic capacity that contributes to their survival and growth in oxic environments (Lin et al., 2004). Thus, environmental surveys cannot solely rely on the presence of *Geobacter*-like sequences as a proxy of electricigenic activity, and need to consider genes encoding proteins involved in oxygen tolerance such as superoxide reductase (Jenney Jr. et al., 1999), superoxide dismutase (Nakanishi et al., 2003), and NADH oxidase (Ward et al., 2001). This is of particular relevance to planted systems such as HSSF CW, which comprise a macrophytes root system that continuously releases oxygen in the surrounding

medium (Tanner, 2001). Moreover, plant evapotranspiration, which is needed to supply oxygen to the cathode in CW-MFCs (Doherty et al., 2015), causes daily fluctuations of the water level that favor oxygen intrusions in the gravel media and increase the redox potential in some areas of the treatment bed (Pedescoll et al., 2013a). This is expected to reduce the representation in the anode biofilms and/or electrochemical activity of oxygen-sensitive electricigens, such as *G. lovleyi*, and reduce the performance of CW-MFCs.

In addition to the low oxygen tolerance of *G. lovelyi*, power generation in CW-MFCs is likely limited by the inability of this organism to use reduced electron donors that are abundant in CW. Indeed, we demonstrated that growth of *G. lovleyi* was supported with acetate as electron donor but lactate and formate could not be utilized. Acetate is a key metabolic intermediate in anaerobic digestion and an abundant electron donor in HSSF CW. In principle, the acetate rich environment in HSSF CW is predicted to promote the growth and sustain power production by *Geobacter* electricigens. However, the electrochemical activity of *G. lovleyi* in anode biofilms is limited by its inability to grow saturating biofilms on the electrode (Figure 4.4.4). Furthermore, optimal current generation by *G. lovleyi* required the anode to be poised at a sufficiently high potential (450 mV vs. SHE). Such operational parameters are difficult to implement in HSSF CW, where redox gradients fluctuate widely in response to external conditions (Corbella et al., 2014). Redox potentials in deep zones of planted HSSF CW can reach ca. -200 mV vs SHE, and generate a maximum voltage of 140 mV (Corbella et al., 2014). This suggests that the anode potential in CW-MFCs is negative and, thus, suboptimal for the growth and electroactivity of *G. lovleyi* (Figure 4.4.4). Studies (van den Berg et al., 2015) show that *G. lovleyi*-like sequences can be enriched at high acetate-to-nitrogen ratios, as these are conditions that stimulate the growth of dissimilatory nitrate reduction to ammonium (DNRA) with the ability to oxidize acetate. Hence, improved CW-MFC performance needs to consider pretreatment approaches that maintain acetate:nitrate ratios such that enrich for non-DNRA, and perhaps oxygen-tolerant, *Geobacter* electricigens so they can become the dominant electricigenic population in the anode biofilms.

Improving domestic wastewater treatment efficiency

Abstract

For the past few years, there has been an increasing interest in the operation of constructed wetlands as microbial fuel cells (CW-MFCs) for both the improvement of wastewater treatment efficiency and the production of energy. However, there is still scarce information on design and operation aspects to maximize CW-MFCs, especially for the treatment of real domestic wastewater. The aim of this study was to quantify the extent of treatment efficiency improvement carried out by membrane-less MFCs simulating a core of a shallow horizontal subsurface flow constructed wetland. The influence of the external resistance (50, 220, 402, 604 and 1000 ohms) and the anode material (graphite and gravel) on treatment efficiency improvement were addressed. To this purpose, 6 lab-scale membrane-less MFCs were set-up and loaded in batch mode with domestic wastewater for 13 weeks. Results showed that 220 ohms was the best operation condition for maximising MFCs treatment efficiency, regardless the anode material employed. Gravel-based anode MFCs operated at closed circuit showed ca. 21%, 18%, 15%, 31% and 25% lower effluent concentration than unconnected MFCs to the BOD_5, COD, TOC, PO_4^{-3} and NH_4^+-N, respectively. Main conclusion of the present work is that constructed wetlands operated as MFCs is a promising strategy to improve domestic wastewater treatment efficiency. However, further studies at pilot scale under more realistic conditions (such as planted systems operated under continuous mode) shall be performed to confirm the findings here reported.

5.1 Introduction

Microbial Fuel Cells (MFCs) are bioelectrochemical devices that generate electricity from organic matter oxidation by means of exoelectrogenic bacteria as catalysts (Logan, 2008). In a MFC, organic matter is oxidised in the anodic compartment and electrons resulting from the oxidation are transferred to the electrode (anode) from where they flow through a conductive material and a resistor to reduce a higher electron acceptor, such as oxygen, at the cathode (Rabaey and Verstraete 2005). Therefore, to produce energy with a MFC enough redox gradient must exist between the anode and the cathode. To provide enough redox gradient MFCs are generally designed with a membrane permeable to protons (PEM) between the anode and the cathode (Logan et al., 2006). However, there are other MFCs configurations that exploit the naturally generated redox gradient in aquatic environments (such as rice paddy fields and marine sediments) that do not require the presence of a PEM (Bond et al., 2002; Kaku et al., 2008). This type of membrane-less MFCs are usually known as sediment MFCs. Recently, there has been an increasing interest on the application of membrane-less MFCs in constructed wetlands, especially in horizontal subsurface-flow configurations (HF CWs) (Doherty et al., 2015) either for the improvement of wastewater treatment efficiency or the energy production.

HF CWs are natural wastewater treatment systems in which pollutants are removed by means of physical, chemical and biological processes (García et al., 2010). They are capable of treating wastewater from a wide range of origins such as urban, industrial or agricultural (Kadlec and Wallace, 2009). Due to their low energy requirements and their easy operation and maintenance, HF CWs have become an alternative to conventional intensified systems for the sanitation of small communities (Puigagut et al., 2007b). They consist of permanently flooded planted gravel beds that are considered to be mainly anaerobic (Baptista et al., 2003). Therefore, the oxidation of the organic matter within these systems is mainly carried out by means of anaerobic degradation pathways, which are characterized to be slower and less efficient than aerobic ones. Due to its anaerobic nature, HF CWs have relatively larger surface requirements when compared to traditional intensive technologies such as activated sludge systems. During the last years, research in HF CWs has focused on the improvement of treatment performances and

its consequent reduction of surface requirement. Forced (or active) aeration of CWs has been recently suggested as a suitable strategy to improve the removal of organic matter and nitrogen species (Nivala et al., 2013; Wu et al., 2014). However, in spite of the advantages of aeration strategies, its higher energy requirements result in higher costs of operation. Therefore, there is a current need for innovative wetlands configurations that provide an increase in treatment efficiency while keeping the energy consumption as low as possible.

In HF CWs, redox potential decreases with depth generating a vertical redox gradient (García et al., 2003; Pedescoll et al., 2013a) between the upper zone of the treatment bed, which is in higher redox conditions due to the oxygen diffusion from the atmosphere and the oxygen from plant supply, and the deeper zones where the anaerobic environment predominates. Therefore, HF CWs can be exploited to produce energy via MFCs (Corbella et al., 2016b, 2014). In MFCs implemented in HF CWs the anode is placed in the anaerobic area while the cathode is placed in contact with the atmospheric oxygen (Corbella et al., 2016b). Therefore, the anode constitutes an inert and non-consumable electron acceptor with a high redox potential located in a very anaerobic environment. Under this configuration bacteria would gain more energy when using the anode as the electron acceptor in comparison to the commonly used electron acceptors present in HF CWs like nitrates or sulfates, and overall treatment efficiency increases (Srivastava et al., 2015).

In spite of the envisaged improvement in treatment efficiency derived from the operation of a constructed wetland as a microbial fuel cell, there is still little information on design and operation aspects that would lead to an enhancement of treatment efficiency, especially for the treatment of real domestic wastewater.

The aim of this study was to quantify the extent of treatment efficiency improvement in lab-scale membrane-less MFCs simulating a core of a shallow wetland. The experimental MFCs were loaded with primary settled domestic wastewater and their treatment efficiency was monitored as function of the external resistance used (50, 220, 402, 604 and 1000 ohms) and the material of the anode employed (graphite and gravel). Although the experimental systems here used cannot be fully considered as constructed wetlands due to

a lack of plants and the batch loading regime, results here reported will be still of use towards an improvement on the design and operation of constructed wetlands working as microbial fuel cells.

5.2 Materials and methods

5.2.1 MFC configuration

For the purpose of this work 6 lab-scale membrane-less microbial fuel cells (MFCs) were operated for 13 weeks (Figure 5.1). At the moment of the experimental campaign all MFCs had been previously loaded with domestic wastewater for 1 year producing a stable electric signal and thus guaranteeing the development of a mature biofilm. According to the objective of the study, the anode chamber of some of the experimental MFCs used was designed to simulate a core of a shallow wetland. Therefore, anodes of 4 of the 6 systems employed were filled with granitic gravel of 0.8 cm in diameter (D60=7.3; Cu=0.8; porosity=40%). Two of the gravel-based MFCs were operated at closed circuit conditions (MFC$^+$) and two of them were operated at open circuit conditions (MFC$^-$). In order to test the influence of the anode material used the other 2 experimental MFCs, which were also operated at closed circuit conditions, had a graphite based anode (made with graphite rods of 1 cm length and 0.5 cm diameter).

Each MFC consisted of two chambers (the anodic and cathodic chambers) (Figure 5.1). The anodic chamber was made of a PVC cylinder of 9 cm diameter and 15 cm of height filled with graphite or gravel. The anode chamber had a total working volume of 0.5L. Both materials were wrapped in a stainless steel mesh (marine grade 316L) that worked as an electron collector every 4 cm. Stainless steel mesh is described to be one of the main contributors to both the environmental impact (abiotic depletion) and to the capital costs of constructed wetlands coupled with MFC (Corbella et al., 2017) and therefore its introduction to the system must be minimized. In order to ensure adequate mixing conditions, water inside the anode chamber was continuously recirculated by means of a peristaltic pump (Damova MP-3035-6M; Toshiba VF-nC3). The cathode chamber consisted of a PVC cylinder placed just above the anode chamber filled up with 4 pieces of graphite felt giving a total surface area of 61 cm^2. Each piece of graphite felt was provided

by Alfa Aesar (1.12 cm thick, 99.9 %; metal basis). Graphite pieces of the cathode were inter-connected using stainless steel wires (marine grade 316L). A layer of glass wool was placed between the anode and the cathode chamber so as to avoid any oxygen leaking from the cathode as recommended elsewhere (Venkata Mohan et al., 2008). The anode and the cathode were externally connected by means of copper wires through an external resistance that ranged from 50 to 1000 ohms during the first part of the experiment (first 6 weeks) and then was finally to at 220 ohms for the rest of the study period.
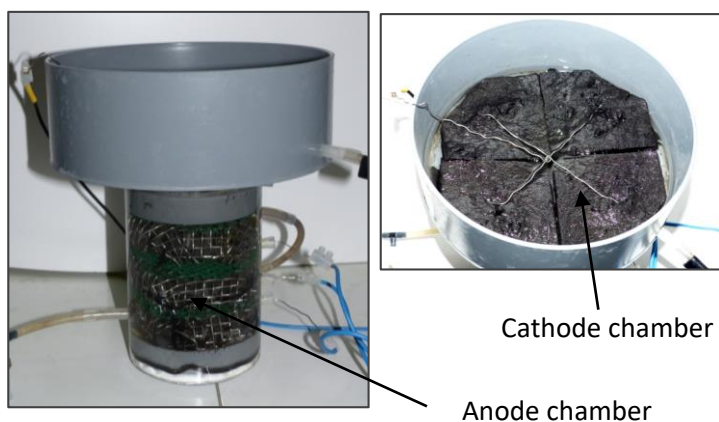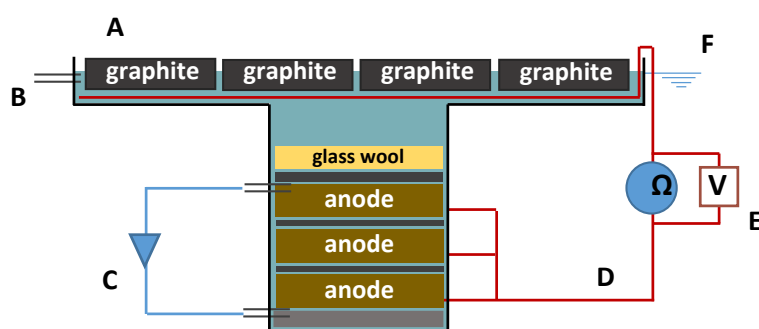
Figure 5.1 Schematics (up) and pictures (down) of experimental MFCs used. A) Graphite felt cathode; B) Outflow to guarantee a constant water level within the system; C) Recirculation pump; D) External electric circuit; E) Data logger to measure and register the voltage across the external resistance; F) Water level within the system

5.2.2 Experimental design

External resistance is one of the most influencing parameters affecting MFCs performance (Aelterman et al., 2008). Therefore, an experimental campaign was initially carried out in order to determine the optimal external resistance that maximized MFCs treatment efficiency. To this aim, 4 gravel-based anode MFCs (2 connected and 2 unconnected) were operated during 6 weeks. Systems were loaded in batch mode every 24 hours with primary settled domestic wastewater that was collected and frozen at the beginning of the experiment and was defrosted every day before the feeding. Five different external resistances were tested: 50, 220, 402, 604 and 1000 ohms. Each one of the experimental MFC was operated for one week under a selected external resistance and influent and effluent water quality was monitored three times along the week. After one week the external resistance was changed to a new value and the system was left for about 24-48 hours until the electrical signal was stable under the new implemented external resistance. This procedure was repeated for all the tested external resistances considered. Soluble and total COD from inlet and effluent samples were analyzed according to APHA (2005). The optimal working conditions were selected by choosing the external resistance that maximized the organic matter difference between the effluent of the connected (MFC$^+$) and non-connected (MFC$^-$) systems.

Once the optimal resistance was determined, the experiment to assess the actual capacity of MFCs to improve CWs' treatment efficiency on a wider range of contaminants was carried out. To this purpose, 6 lab-scale MFCs were operated during 7 weeks. These MFCs consisted of 4 gravel-based MFC used in the first experiment plus 2 graphite-based MFCs that had been equally fed during the first experiment but not monitored regarding treatment efficiency. During this second experiment, MFCs were weekly fed with primary settled domestic wastewater and samples were taken at the inlet and at the outlet of the anode chamber of each cell after 7 days of contact time. To determine the effect of MFCs on the improvement of CWs' treatment efficiencies results from connected (MFC$^+$) and non-connected (MFC$^-$) gravel-based MFCs were compared. On the other hand, to assess the effect of the anodic material on removal rates, results from connected gravel and graphite-based MFCs were compared. Each experimental condition was tested in duplicate.

5.2.3 *Physical-chemical analysis and electrical monitoring*

Water quality parameters analyzed during the second experiment were total and soluble organic carbon (TOC_{tot} and TOC_{sol}), biochemical oxygen demand (BOD_5), total and soluble chemical oxygen demand (COD_{tot} and COD_{sol}), ammonium, nitrates and nitrites (NH_4^+-N, NO_3^--N, NO_2^--N) , sulphates (SO_4^{2-}-S) and ortophosphates (PO_4^--P). Water quality parameters were analyzed following standard methods (APHA, 2005). Table 5.1 summarizes water quality parameters during the experiment.

Voltages generated by the 4 connected MFCs (2 of them with graphite-based anodes and the other 2 with gravel-based anodes) were recorded across the external resistance and stored every minute by means of a datalogger (Campbell Scientific CR1000). Current densities, power densities and coulombic efficiencies were calculated according to Logan et al. (2006).

Table 5.1 Physico-chemical parameters analyzed (TOCtot, TOCsol, CODtot, CODsol, BODtot, SO42--S, NH4+-N, NO3-N, NO2--N, PO4-P) at the influent and effluent of the graphite and gravel based connected MFCs and the gravel based non-connected MFC. Also removal rates are presented

			IN	EFFLUENT		
			-	GRAPH	GRAV	
			-	MFC$^+$	MFC$^+$	MFC$^-$
			mg/L	mg/L	mg/L	mg/L
TOC	TOC tot	MEAN	239.8	16.0	18.4	22.2
		SD	43.6	3.5	4.0	6.7
	TOC sol	MEAN	62.3	17.5	16.5	20.1
		SD	21.5	6.7	4.7	4.9
COD	COD tot	MEAN	625.1	107.8	112.1	134.4
		SD	169.8	28.5	25.6	24.2
	COD sol	MEAN	198.0	73.3	64.4	90.1
		SD	31.3	23.1	21.9	20.2
BOD$_5$		MEAN	239.3	134.9	132.7	163.4
		SD	96.6	29.2	33.0	41.7
SULPHATE-S		MEAN	23.4	12.1	10.6	7.9
		SD	2.5	4.1	3.9	2.3
AMMONIUM-N		MEAN	50.4	16.2	15.9	21.2
		SD	6.5	8.0	7.4	8.7
NITRITE-N		MEAN	0.0	0.2	0.2	0.5
		SD	0.0	0.3	0.3	1.0
NITRATE-N		MEAN	0.2	1.7	1.6	1.2
		SD	0.3	0.6	0.5	0.7
ORTOPHOSPHATE-P		MEAN	4.8	3.8	3.6	5.2
		SD	0.6	0.6	0.7	0.7

Note: MFC+: connected CW-MFC; MFC-: non-connected CW-MFCs; IN: influent; GRAPH: graphite-based CW-MFCs; GRAV: gravel-based CW-MFCs

5.2.4   X-ray diffraction analysis

At the end of the study period, the cathode was sampled and subjected to X-ray difraction analysis to determine which compounds had been precipitating during MFC operation. To this purpose, 1g graphite felt pieces of the cathode of connected and un-connected systems were taken and processed as follows.

Sample processing. Samples were ground in an Agate mortar. The resulting powder materials were placed back loaded in cylindrical cavities, of 16 millimetres of diameter and 2.5 millimetres of thickness, of standard sample holders (PW1811/16).

Instrument and analytical conditions.

- PANalytical X'Pert PRO MPD Alpha1 powder diffractometer (radius = 240 millimetres)
- Cu Kα1 radiation ($Λ$ = 1.5406 Å).
- Work power: 45 kV – 40 mA.
- Focalizing Ge (111) primary monochromator
- Sample spinning at 2 revolutions per second
- Variable automatic divergence slit to get an illuminated length in the beam direction of 10 millimetres.
- Mask defining a length of the beam over the sample in the axial direction of 12 millimetres
- Diffracted beam 0.04 radians Soller slits
- X'Celerator Detector: Active length = 2.122 °.
- ɵ/2ɵ scans from 4 to 80° 2ɵ with step size of 0.017° and measuring time of 50 seconds per step.

Determination. The determination method was that of the qualitative crystalline phase analysis by means of the PDF (Powder Diffraction File) data base of the ICDD-JCPDS (International Centre for Difraction Data – Joint Cometee of Powder Diffraction Standards, 2015).

5.2.5 *Statistical analysis*

Differences among experimental conditions for all the considered parameters (physical-chemical and electrical parameters) were determined by carrying out an ANOVA test of variance. Homogeneity of variances and normality of data were assessed by performing a Levene test and a Kolmogorov–Smirnov test, respectively. Differences among experimental conditions were considered significant at p values bellow 0.1; p values below 0.05 were also indicated. All statistical analyses were performed using the software package R 3.0.2.

5.3 **Results and discussion**

5.3.1 *Determination of the optimal external resistance*

The relationship between the current and the voltage generated is determined by the external resistance (Logan et al., 2006). Different configurations (materials, dimensions, substrates, etc.) are linked to different internal resistances and hence, different optimal external resistances (Logan, 2008). Furthermore, depending on the purpose of the experiment, the control of the external resistance could foster either the power produced or the wastewater treatment (Katuri et al., 2011). Overall, it is generally accepted that the lower the external resistance the higher the current generated and the higher the organic matter removal rate (Aelterman et al., 2008; Gil et al., 2003; Katuri et al., 2011).

Outlet COD concentrations in our MFC$^+$ were generally lower than that of MFC$^-$ (Figure 5.2 A). However, the highest significant differences (p<0.05) between connected and not-connected systems were recorded at 220 ohms of external resistance either for total or soluble COD (Figure 5.2 A). Furthermore the difference between MFC$^+$ and MFC$^-$ outlet concentrations tended to decrease with the external load applied (Figure 5.2 B). These results are in accordance to Katuri et al. (2011) who reported an increase of organic matter removal of about 30% under lower external resistance (100 ohms) when compared to higher external resistance (50,000 ohms). In line with this, Song et al. (2010) reported higher organic matter removal efficiencies at a resistance of 100 ohms when a range between 10 and 1000 ohms was tested

in freshwater sediment MFCs. In their study, the calculated internal resistance was determined to be higher when the external resistance was of 10 ohms than when it was of 100 ohms leading to lower current and removal efficiencies. Overall, and according to our results, 220 ohms was the external resistance chosen for the following experiments that aimed at determining to which extent MFCs improve treatment efficiency for a wide range of contaminants.
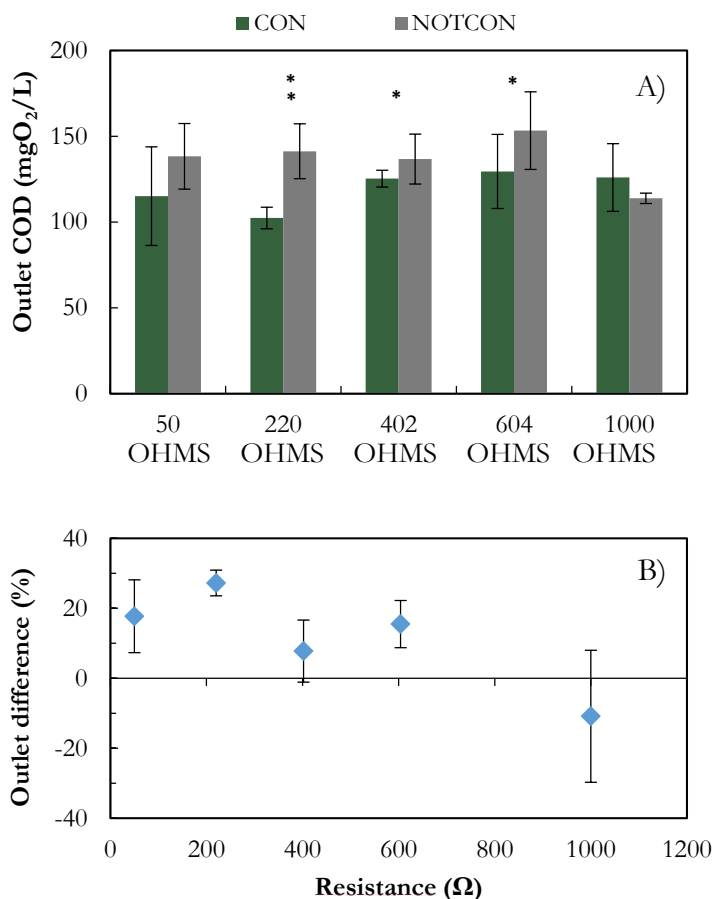
Figure 5.2 Effect of MFCs external resistance on MFC treatment efficiency improvement. A) Outlet soluble COD concentrations in connected (CON) and not-connected (NOTCON) MFCs as function of the external resistance. B) Difference between connected and not-connected effluents ($COD_{NOTCON} - COD_{CON}$) in terms of total COD concentration. Statistical significances are indicated: ** : $p_{value} < 0.05$; * : $p_{value} < 0.1$. *Note*: for each bar/dot depicted in Figure 5.2 n=6

5.3.2 Effect of anodic material on treatment efficiency and electrical output

The electrode material used in MFCs architecture is a key parameter regarding their performance. Good electrical conductivity is one of the most important properties to optimize MFCs functioning (Zhou et al., 2011). Therefore, non-conductive materials are rarely used as anodic materials in conventional MFCs due to its incapacity to collect and transfer electrons. In this study we compared the effect of using a very common conductive electrode material (graphite) with a non-conductive material typically described as filling media in constructed wetlands (gravel). Previous studies of our group demonstrated the feasibility of using gravel as anodic material when an electron collector is provided (stainless steel mesh) (Corbella et al., 2016a). In that case, the gravel matrix did not transfer the electrons itself but provided a physical surface for the electrical conductive biofilm to stablish. (Corbella et al., 2015a).

Unexpectedly, graphite and gravel-based anodes of MFCs did not produce any significant difference on the removal efficiency for any of the analyzed water quality parameters (Figure 5.3 – Table 5.1). MFCs used in this experiment were previously operated during 1 year before the experiment was conducted. Domestic wastewater is known to have high content of suspended solids (Pedescoll et al., 2011b) which progressively accumulates within the system. This solids accumulation has been demonstrated to decrease MFCs performance in gravel based systems (Corbella et al., 2016a). Accordingly, the authors of the present paper believe that the accumulation of solids within the systems might have decreased the capacity of the system to transfer electrons to the external circuit to the point that no differences can be detected between a conductive media such as graphite and a non-conductive media such as gravel based MFCs. Therefore, it remains still unknown whether at early stages of MFC functioning graphite-based electrodes would outperform non-conductive media in terms of pollutants removal.
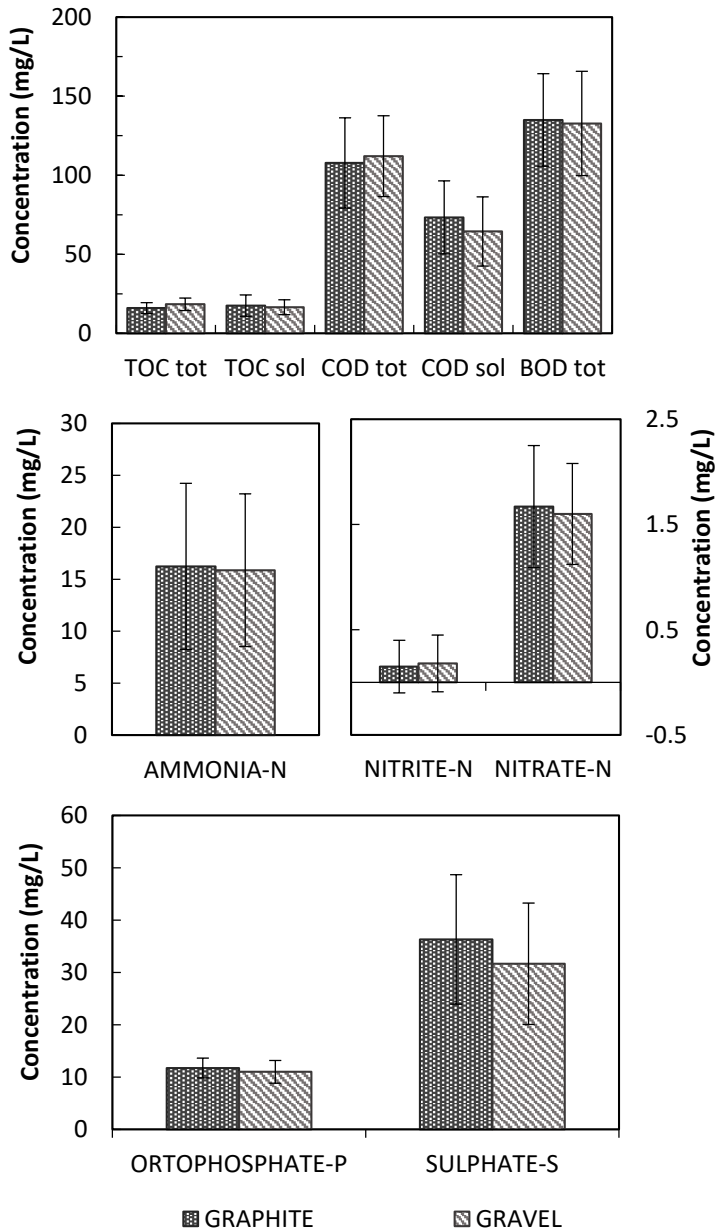
Figure 5.3 Effluent concentrations of physical-chemical parameters surveyed for graphite and gravel-based MFCs. *Note*: statistical analysis was performed and no differences were found between materials regardless the parameter considered

The same conclusion as above can be stated regarding cell voltage recorded as function of the anode material. Mean voltages obtained all along the experiment were 123±49 mV and 102±60 for gravel and graphite-based systems, respectively. Furthermore, maximum voltages obtained were also very similar among tested anode materials (238 and 245 mV for gravel and graphite basted CW-MFCs, respectively). The same trend was observed in terms of weekly charge produced (305±134 C and 253±88 C, for gravel and graphite MFCs, respectively). Similar maximum power densities were recorded for gravel and graphite based MFCs (288 and 346 mW/m^3 CW, respectively). Also similar maximum current densities were found (1.17 A/m$^3_{anode}$ and 1.28 A/m$^3_{anode}$ for gravel and graphite based MFCs). Overall, these results are in the range of those reported for CW-MFCs set under different configurations (Doherty et al., 2015; Oon et al., 2015; Srivastava et al., 2015).

Due to the fact that no differences were found between the anode materials here considered, only results from gravel based MFCs are presented below. Therefore, results shown from this moment on compare treatment efficiencies between connected (MFC$^+$) and non-connected (MFC$^-$) gravel-based MFCs.

5.3.3 Organic matter removal

Average coulombic efficiencies (CE) were 10±6% and 42±14% for total and soluble COD, respectively. Such CEs are common in MFCs loaded with complex organic substrates such as the wastewater here employed (Larrosa-Guerrero et al., 2010). Reported CE values for MFCs implemented in wetlands are actually lower than those here reported (between 0.05% and 3.9%) (Doherty et al., 2015). Furthermore, our results also agree with that previously stated in literature regarding higher CE for soluble COD than for total COD (Huang et al., 2011). This is due to the fact that exoelectrogenic bacteria are only able to oxidize simple carbohydrates. Therefore, particulate organics need to be hydrolyzed to volatile fatty acids before being utilized by exoelectrogens (Kiely et al., 2011). Moreover, physical removal of particulate organic matter by filtration in MFCs contributes to total OM removal but it is just a physical-based removal process, thus lowering the total OM CE.

Organic matter removal efficiency in this study was assessed by means of different parameters such as BOD_5, total and soluble COD and total and soluble organic carbon (OC). The concentration of organic matter at the effluent of MFC^- was always statistically higher than in MFC^+, indicating a stimulation of the organic matter oxidation in connected systems (Table 5.1 – Figure 5.4). More precisely, effluent concentrations in connected systems were 21±2 %, 18±11% and 15±16% lower than not-connected systems, for BOD_5, COD and total OC, respectively. These results are in accordance to current literature where an improvement on treatment efficiency of MFCs operated at closed circuit conditions has been described for organic substrates such as glucose (Srivastava et al. 2015) and synthetic wastewater (Oon et al., 2016). Furthermore, sediment and conventional MFCs have been also described to improve anaerobic degradation of organics (Huang et al., 2011; Larrosa-Guerrero et al., 2010).

Figure 5.4 Influent (IN) and effluent (OUT) concentrations in connected (CON) and non-connected (NOCON) gravel based MFCs for the organic matter parameters analyzed: total and soluble organic carbon (TOC_{tot} and TOC_{sol}), total and soluble biochemical oxygen demand (COD_{tot} and COD_{sol}) and biological oxygen demand (BOD_{tot}). Statistical differences between effluent concentrations of the CON and the NOCON systems are indicated (* : $p_{value} < 0.1$; ** : $p_{value} < 0.05$) (n= 12 to 27)

Furthermore, results indicate that the equivalent organic matter removed by the exoelectrogenic pathway in connected MFC (estimated from the electrons transferred through the external circuit) is much higher (25.2 ±11.1 mg O_2) than the actual difference between the connected and the unconnected MFCs (average difference being 11.7 ±11.3 mg O_2). Authors suggest two hypotheses for this fact (Figure 5.S1). On the one hand, this result may suggest that there is some inorganic electro-active species that is interacting with the electrode but cannot be detected by means of the COD analysis. The second hypothesis on the other hand is based on the fact that bacteria performing the anaerobic degradation of organic matter such as methane producing bacteria) are being outcompeted by exoelectrogenic bacteria. According to current literature, bacteria that are able to reduce an electrode would gain more energy per mol of organic matter oxidized than anaerobic bacteria (Li and Yu, 2015). Therefore, if exoelectrogenic bacteria are actually outcompeting anaerobic bacteria, the conventional anaerobic pathways for organic matter oxidation in MFC^+ would decrease because of the existence of a more efficient degradation pathway (exoelectrogenesis). According to this second hypothesis, the fact that the COD equivalent to the current generated is higher than the actual difference between the COD removed by MFC^+ and MFC^- is just an artifact. This artifact is caused by the assumption that anaerobic organic matter removal pathways in MFC^+ are of equal extent than those of MFC^- and that the increase of treatment efficiency in MFC^+ is the sum of the COD removed by conventional anaerobic routes plus the exoelectrogenic route. However, if this second hypothesis is true, the actual increase of organic matter treatment efficiency in MFC^+ shall be estimated taking into account that anaerobic routes in active systems are of lesser extent than in MFC^- (Figure 5.S1). Unfortunately, our experimental design does not allow us to differentiate the relative contribution of each one of the two suggested pathways. Therefore, it remains still unknown which is the main route responsible for the improvement of MFC treatment efficiency here reported.

5.3.4 *Nutrient removal: nitrogen, sufphate and phosphorus*

Although the main purpose of constructed wetlands is the removal of organic matter from wastewater, other contaminants such as nitrogen or

phosphorus are also of importance given current figures of environmental pollution and environmental policy restrictions.

In subsurface flow constructed wetlands nitrogen is mainly removed by means of nitrification and denitrification processes (García et al., 2010). While the nitrification requires oxygen and low carbon contents, denitrification requires carbon and an anoxic environment. In horizontal subsurface flow constructed wetlands, nitrification is the main limiting process for overall nitrogen removal due to restricted oxygen availability within the treatment bed. However, depending on environmental conditions other nitrogen removal pathways (such as anaerobic ammonia oxidation) can occur (Pelissari et al., 2016; Saeed and Sun, 2012). Ammonia removal in our experiment was on average 66±14% and 53±17% for MFC$^+$ and MFC$^-$, respectively (Table 5.1). These values are within the range of those reported in the current literature for the treatment of swine slurry with MFCs (Doherty et al., 2014). Nitrite and nitrate species were also found in MFC$^+$ and MFC$^-$ outlet samples, confirming that nitrification was being carried out within the systems as has been already described for wetlands operated under batch hydraulic regime (Pedescoll et al. 2011b). However, nitrification is an aerobic process that requires 4.3 g of O_2 per gram of NH_3-N oxidized (Metcalf and Eddy, 1991). Oxygen concentrations within the anodic chamber ranged from <0.1 to 0.4 mg O_2/L. Nitrification has been described to be a possible pathway at low oxygen concentrations (Fitzgerald et al., 2015). However, in such high carbon and low oxygen content environment the aerobic heterotrophic pathway would predominate over the nitrification process when competing for oxygen. Under these conditions, nitrification probably occurred within the cathodic chamber where wastewater was in direct contact with the atmosphere. Recirculation of wastewater within the anodic chamber probably enabled the subsequent denitrification within the anodic compartment (Oon et al., 2016).

Furthermore, results showed that ammonia outlet concentration was always lower in MFC$^+$ when compared to the MFC$^-$ (P<0.1), indicating a stimulation of the ammonia removal in connected systems (Figure 5.5). This result is in accordance to that described in current literature (Jung et al., 2008; Lu et al., 2009). Higher ammonium removal in active MFCs has been mainly attributed to an increase of pH near the cathode as a consequence of the

consumption of protons which results in direct volatilization of ammonia. However, our pH measurements within the bulk liquid surrounding the cathode ranged between 6.5 and 7.2, regardless the experimental system considered (MFC$^+$ vs MFC$^-$); therefore, we can hardly think of ammonia volatilization being the predominant process for higher ammonia removal in our systems. Furthermore, active microbial fuel cells have been also described to enhance the presence of anaerobic ammonia oxidation bacteria (ANAMMOX) (Di Domenico et al., 2015). Anammox process has much lower oxygen requirements than the conventional nitrification/denitrification process. Accordingly, in our experimental systems oxygen concentration was very low which may have resulted in an adequate environment for the anammox process to take place. Unfortunately, no microbial population studies were performed in our experimental systems. Therefore, still remains unknown whether the overall ammonia treatment improvement observed in our active MFC was related to and enhancement of anammox populations.
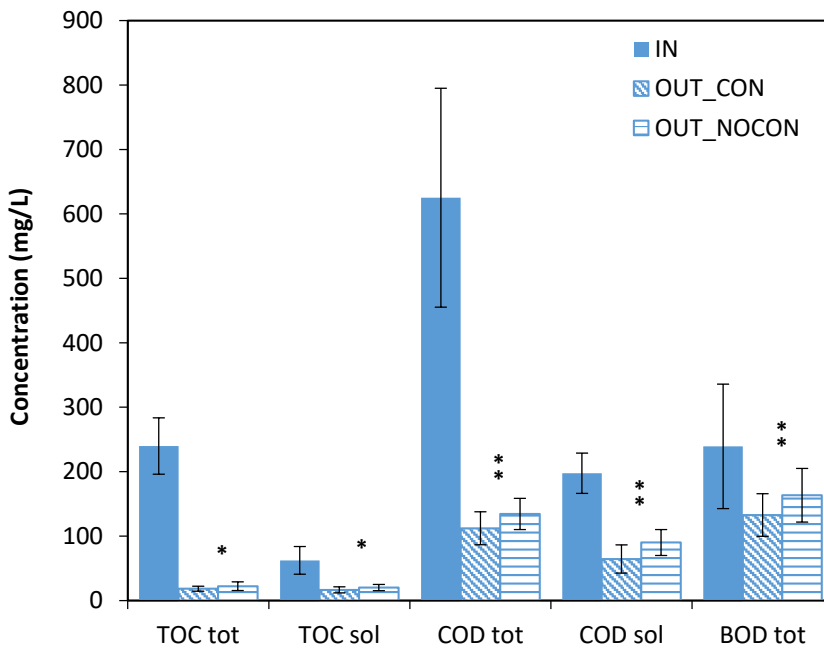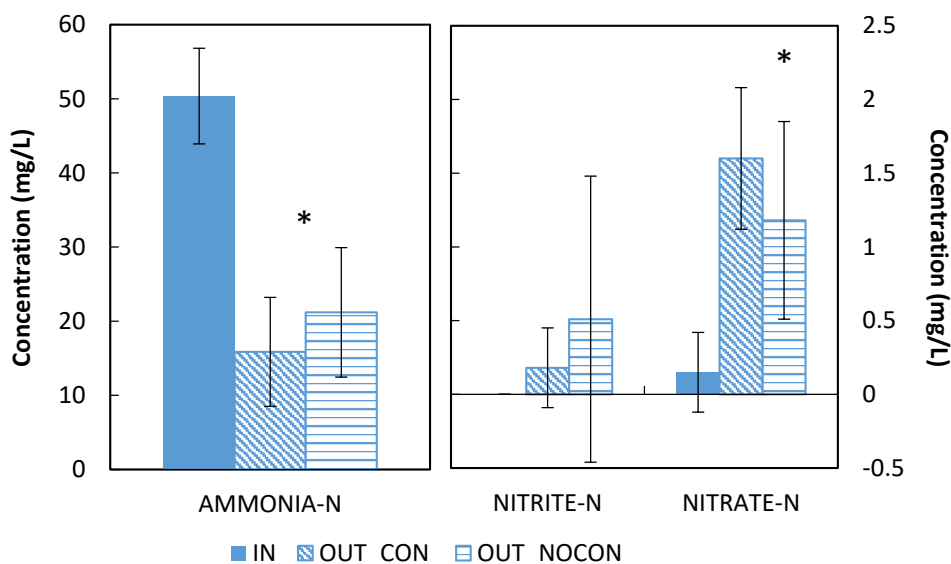

Figure 5.5 Influent (IN) and effluent (OUT) concentrations at connected (CON) and non-connected (NOCON) gravel based CW-MFCs for the nitrogen compounds analyzed: ammonia (NH_4^+-N), nitrite (NO_3^--N) and nitrate (NO_2^--N). Statistical differences between effluent concentrations of the CON and the NOCON systems are indicated (* : $p_{value} < 0.1$; ** : $p_{value} < 0.05$; n=12)

Sulfate concentration was also monitored along the study period, and results are presented in Figure 5.6. Inlet wastewater used in this experiment constituted a sulphate-rich media. However, although not connected systems were able to remove up to 70% of the inlet sulphate, it was less efficiently removed in connected systems (ca. 57%). According to current literature, sulfides (that are the product of the sulfate reduction through the oxidation of the organic matter by means of sulfate reducing bacteria) can be partially re-oxidized to sulfur using the anode as the electron acceptor, thus generating electrical current. Then sulfur is described to be potentially further oxidized into sulfates in a cyclical sulfate-regenerating process (Lovley, 2006). Therefore, if sulfates are being regenerated in our system, their concentration should be higher in connected than in unconnected MFCs. Our results support this hypothesis since the concentration of sulfates in the anode chamber of connected MFCs was always significantly higher than those measured in unconnected MFC (Figure 5.6).
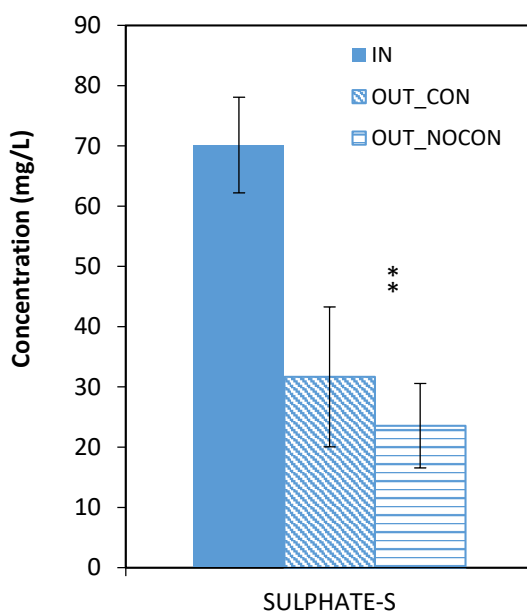
Figure 5.6 Influent (IN) and effluent (OUT) sulphate (SO_4^{2-} - S) concentrations in connected (CON) and non-connected (NOCON) gravel based MFCs. Statistical differences between effluent concentrations of the CON and the NOCON systems are indicated (* : $p_{value} < 0.1$; ** : $p_{value} < 0.05$; n=12)

Phosphorus removal in our active MFC was significantly increased when compared to unconnected MFC, and accounted for about the 20% of the phosphorus influent concentration (Figure 5.7). The enhancement of ortophosphates removal in MFC[+] systems has been reported in previous studies and the main mechanism described is the formation of phosphorus precipitates around the cathode in the form of struvite that can account for up to the 27% of the influent phosphorus concentration (Ichihashi and Hirooka, 2012). In our experiment we also found a white precipitate around the cathode (Figure 5.S2). However, X-ray diffraction analysis technique did not reveal any phosphorus-based compound, and the precipitates observed at the cathode were mostly Calcite ($CaCO_3$) and Halite ($NaCl$), regardless the experimental condition considered (Figure 5.S3). It has been also described that biological phosphorus uptake can be enhanced in bioelectrochemical systems working under low current conditions (<10 A) (Zhang et al., 2012). The average current generated in our systems was very low (ca. 1.45 mA and 1.6 mA to the gravel and graphite-based MFC, respectively), which might have enhanced the phosphorus removal through biological uptake rather than chemical precipitation at the cathode. Overall, authors believe that future work on the characterization of biological phosphorus uptake in membrane-less microbial fuel cells working under low current generation conditions shall be addressed to confirm the findings here reported.
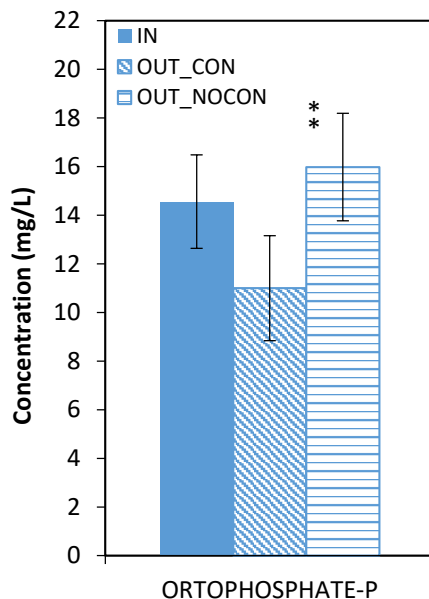
Figure 5.7 Influent (IN) and effluent (OUT) orthophosphate (PO_4^- - P) concentrations at connected (CON) and non-connected (NOCON) gravel based MFCs. Statistical differences between effluent concentrations of the CON and the NOCON systems are indicated (* : p_{value}<0.1; ** : p_{value}<0.05; n=12)

5.3.5 Significance of results reported in the context of constructed wetlands

Results here reported strongly suggest that the operation of constructed wetlands as MFCs would increase domestic wastewater treatment performance. Constructed wetlands (CWs) is a robust and efficient technology that, when properly designed, is able to meet the limits established in current environmental policy (Kadlec and Wallace, 2009). However, CWs are suitable technologies only for small communities (Puigagut et al., 2007b) due to their large surface requirements when compared to conventional wastewater treatment technologies (Kadlec and Wallace, 2009). Accordingly, current research efforts on wetland technology have focused on the design and implementation of intensive strategies (such as wetlands aeration, reciprocation, specific filter media, etc.) to improve nutrients and organic

matter treatment efficiency and reduce surface requirements. However, most of the currently available intensified wetlands configurations result in higher costs of operation (mostly due to higher energy consumption rates). In this context, results here reported point out MFCs as an alternative to currently available intensive HF CWs strategies to increase their removal rates with energy generation instead of high energy consumption. Furthermore, higher organic matter degradation rates in HF CWs operated as MFCs may result in a reduction of wetlands' surface requirements, thus enabling more compact designs and reducing the cost for land acquisition (Srivastava et al., 2015).

Overall, our results suggest that HF CWs operated as MFCs is a promising strategy to improve domestic wastewater treatment efficiency (either in terms of organic matter removal or nutrients). However, further studies at pilot scale under more realistic conditions (such as planted systems operated under continuous flow) shall be performed to confirm the findings here reported.

5.4 Conclusions

220 ohms is the best external resistance out of those here tested to optimize the treatment of domestic wastewater with constructed wetlands membrane-less microbial fuel cells.

For the purpose of improving domestic wastewater treatment efficiency, gravel or graphite-based anodes coupled to an electron collector (stain-less steel mesh) appear to be equally suitable.

Gravel-based anode membrane-less MFCs operated at closed circuit showed ca. 21%, 18%, 15%, 31% and 25% lower effluent concentration than unconnected MFCs to the BOD_5, COD, TOC, PO_4^{-3} and NH_4^+-N, respectively.

Constructed wetlands operated as MFCs is a promising strategy to improve domestic wastewater treatment efficiency. However, further studies at pilot scale under more realistic conditions (such as planted systems operated under continuous mode) shall be performed to confirm the findings here reported.

COD and clogging assessment with CW-MFCs

In this chapter all the investigations concerning the capacity of CW-MFCs to assess de COD content in wastewater and the clogging state within CWs treatment bed are gathered. The chapter is divided in two different sections corresponding to the two different articles referenced in the previous page. As a consequence, the reader will find the formal structure of an article two consecutive times in this chapter.

Novel membrane-less MFC-based biosensor for domestic wastewater COD assessment in constructed wetlands

Abstract

A microbial fuel cell (MFC) is a technology that enables a bioconversion of chemical energy contained in organic/inorganic compounds into electrical energy. Besides the use of MFCs as a novel technology for green energy production, they can also be used as a biosensor tool for organic matter concentration assessment. In the context of natural-based wastewater treatment technologies (such as constructed wetlands - CW) the use of a biosensor tool for the assessment of operational conditions (such as influent/effluent organic matter concentration) is of key importance for plant management optimization. The objective of the present study was to determine the use of constructed wetland microbial fuel cells (CW-MFCs) as a domestic wastewater COD assessment tool. For the purpose of this work four lab-scale CW-MFCs were set up and fed with domestic wastewater of incrementally increasing COD concentrations. Two different anodic materials were tested (graphite rods and gravel). Results showed that in spite of the low coulombic efficiencies recorded both gravel and graphite-based anodes were suitable for the purpose of domestic wastewater COD assessment. Linear relationships could be established between inlet COD concentrations and CW-MFC intensities (R-squared values higher than 0.9 were found for both gravel and graphite cells). CW-MFC accuracy increased with contact time, regardless the type of anode material considered. However, the accuracy of the CW-MFC was greatly compromised after several days of operation. Although results here reported proof that membrane-less CW-MFC can be of use for domestic wastewater COD assessment, several factors can affect the precision, repeatability and operational stability of the device. Therefore, other

alternatives such as its utilization as a qualitative, range-like assessment tool, should be taken into account when considering practical biosensing applications of CW-MFCs.

6.1.1 Introduction

Organic matter content is one of the legally regulated parameters in wastewater treatment processes. Concentration of organic matter compounds in urban wastewater discharges is limited to 125 mgO$_2$/L of COD by the Spanish legislation (MAGRAMA, 2007). Chemical and biological oxygen demand (COD and BOD, respectively) are the main parameters to determine wastewater organic matter content. Although its precise and rapid quantification is crucial, current methodologies are time consuming, produce chemical compounds that pose a threat to the environment and require qualified personnel (Kumlanghan et al., 2007). As a consequence they are not suitable for real-time monitoring thus preventing a rapid response to contamination events (Di Lorenzo et al., 2009; Kim et al., 2003b). Microbial Fuel Cells are bioelectrochemical systems that generate electricity from the oxidation of organic compounds by means of exoelectrogenic bacteria as catalysts (Logan et al., 2006). Besides the use of MFCs for green energy production, they potentially are a suitable technology for online organic matter concentration assessment. MFCs directly provide an electrical signal that can be correlated to organic wastewater content (Peixoto et al., 2011). The main advantages resulting from the utilization of MFCs as biosensor devices are the possibility of in-situ implementation, on-line monitoring and avoiding complex laboratory procedures requiring addition of chemicals and the fact that no transducer is needed (Di Lorenzo et al., 2009; Peixoto et al., 2011). So far several authors have already proven the viability of using this technology for biosensing purposes both for synthetic and real wastewater COD assessment (Chang et al., 2004; Di Lorenzo et al., 2009).

In the context of natural-based wastewater treatment technologies the use of a biosensor tool for the assessment of operational conditions is of key importance for plant management optimization. CWs are natural wastewater treatment systems in which organic matter is removed by means of physical, chemical and biological processes (García et al., 2010). These removal processes occur within the CWs' treatment bed which is generally made of

gravel and provides the surface for biofilms to establish. Low energy requirements and straightforward operation and maintenance are some of the advantages of CWs (Puigagut et al., 2007b). However MFCs constitute a technology based on high cost materials such as the conductive electrode materials or the cation exchange membranes (Logan, 2008). Therefore, the introduction of a biosensing tool in the context of constructed wetlands needs to address the utilization of both inexpensive and robust materials. In accordance with that, gravel demonstrated to be a suitable surface for some exeoelectrogens to establish (Corbella et al., 2015a) as well as a suitable anodic material when an electron collector (such as stainless steel mesh) is provided (Corbella et al., 2016a). Therefore, the objective of the present study was to assess the use of a novel membrane-less constructed wetland microbial fuel cell (CW-MFC) as a suitable wastewater COD biosensor. Both gravel and graphite were tested as anodic materials. Furthermore, results from a follow-up experiment using pilot-scale CW-MFC systems are also presented.

6.1.2 Materials and methods

6.1.2.1 CW-MFC configuration

CW-MFCs configuration is shown in Figure 6.1.1. Total system volume was 0.85 L. The cylindrical anodic chamber (Ø=9 cm, height=15 cm) was made of transparent acrylic glass and contained three separate layers of the anodic material, each wrapped with stainless steel mesh (marine grade 316L), that served as the electron collector. The three layers of anodic material were externally connected by means of stainless steel wires resulting in a single anodic volume. Two different anodic materials were tested in the experiment: gravel (GRAV-MFC) and graphite rods (GRAPH-MFC). Four replicates of gravel based MFCs and two replicates of graphite based MFCs were constructed. The anodic volume of graphite CW-MFCs was filled with 1 cm long and 6.15 mm wide graphite rods (Alfa Aesar, 99.9995%, metal basis, ACKSP grade, Ultra "F" Purity). Gravel CW-MFCs were filled with granitic gravel commonly used in HSSF CW (D_{60}=7.3; Cu=0.8; porosity=40%). The anodic liquid volume was 0.5 L regardless the anodic material. To guarantee a good homogenization within the anodic volume, wastewater was constantly recirculated by means of a pumping system (Damova MP-3035-6M; Toshiba VF-nC3). A layer of glass wool was placed over the anodic material to avoid

any oxygen leaking from the cathode area. The cylindrical cathodic chamber was made of PVC (Ø=19 cm, height=5 cm). The cathode was assembled using 4 pieces of 60.8 cm^2 graphite felt (Alfa Aesar, 1.12 cm thick, 99.9 % carbon purity; metal basis). As indicated in Figure 6.1.1, graphite felt pieces were placed inside the upper cylinder occupying its entire surface, and were connected in between them and with the external circuit by stainless steel wires. The graphite felt was kept semi-submerged thus in contact with both liquid media and air (air cathode). The anode and the cathode were externally connected with copper wires through an external resistance of 1000 Ω. The generated voltages were measured and stored every 5 minutes across the external resistance by means of a datalogger (Campbell Scientific CR1000).
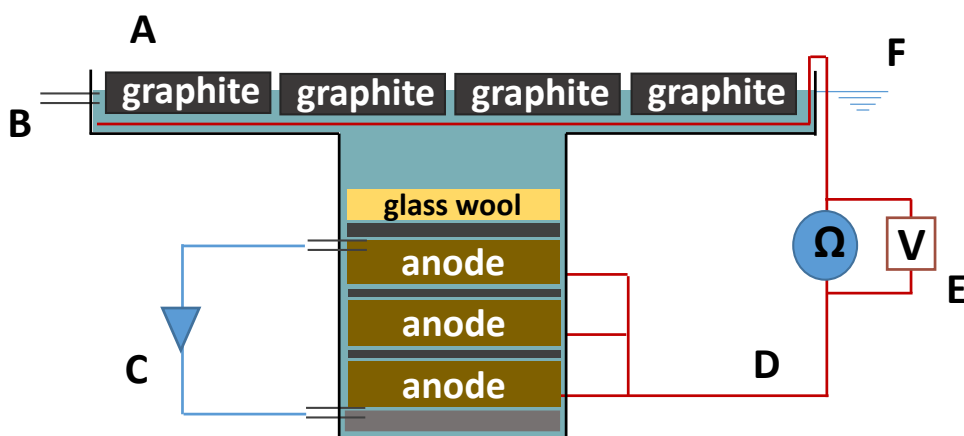
Figure 6.1.1 Scheme of the CW-MFC biosensor. A: graphite felt cathode; B: Cathode overflow; C: Anodic recirculation; D: External circuit; E: Voltmeter and external resistance; F: Water level

6.1.2.2 *Cathode limitation and start-up*

The study's main objective was to investigate the CW-MFC bioindication capacity and aimed at assessing organic matter oxidation processes occurring within the anodic chamber. In order to ensure that cathodic reactions were not limiting the system, i.e. the cathode could cover the anodic current demand, a preliminary experiment was carried out to determine the optimal cathode to anode surface ratio (C:A). Previous experiments of our group determined that the optimal anode to cathode ratio

was 4:1 using graphite as the anodic material (Corbella et al., 2015a). In this case, the experiment was conducted with two MFCs with an anode based on gravel, a non-conductive anodic material. The cathode limitation experiment was conducted by incrementally increasing the cathodic area resulting in the following C:A ratios: 0.8, 1.6, 2.4, 3.2, 4 cm^2cathode/cm^2anode. The cathodic area was increased once the voltage generated by the previous condition was stable for 3h. Voltage was recorded every 5 minutes by means of a datalogger (Campbell Scientific CR1000). After the optimal cathode to anode ratio was determined, cells were cleaned and fed daily with urban wastewater (338±110 mg COD_{tot}/L) for 7 days. This initial feeding period inoculated the systems and generated biofilm until stable voltage values were recorded.

6.1.2.3   *Assessment of current generation by abiotic reactions*

The organic matter bioindication capacity relies on the ability of electrogens to oxidize organic compounds and generate electricity. Bioindication accuracy can be affected by the generation of current stemming from the direct anodic oxidation of non-organic substrates through abiotic reactions. To quantify the electrons generated through abiotic reactions at the anode, an additional specific experiment was carried out. This experiment was performed when the cells had been operated for 6 months, thus the biofilm was stable and well developed within the anodic chamber. The experiment consisted of comparing the electricity generated by biotic and abiotic MFCs. To this aim, two graphite based cells and two of the gravel-based cells were cleaned (intensely with tap water and with a solution of 1N NaOH, Reguera et al., 2011) and sterilized (autoclave, 20 min, 121 ºC, 1 bar) (ABIO_MFCs) while the biofilm of the other two gravel cells was kept unaltered within the anodic chamber (BIO_MFCs). The 6 systems (gravel BIO_MFCs; gravel and graphite ABIO_MFCs) were fed every 48 hours with sterilized wastewater (autoclave, 20 min, 121 ºC, 1 bar) to guarantee an abiotic environment in ABIO_MFCs. This was conducted in triplicate. Between feeding events ABIO_MFCs were cleaned by soaking the anode material in a solution of 1N NaOH thus removing the organic matter and biofilm adhered (Reguera et al., 2011). The generated current was recorded every 5 minutes by means of a datalogger (Campbell Scientific CR1000) during the whole experiment.

6.1.2.4   Bioindication assessment

The bioindication capacity was tested by feeding the systems with wastewater of incrementally increasing chemical oxygen demand (COD) concentrations. Wastewater was collected from the pilot plant described in Corbella and Puigagut (2014), more precisely from the outlet of an anaerobic primary treatment (hydrolytic upflow sludge blanquet reactor, HUSB) which pre-hydrolyses the organic matter present in wastewater (Ligero et al., 2001a). HUSB primary treatment also stimulates the presence of exoelectrogenic bacteria in CW-MFC biofilms (Corbella et al., 2015a). After its collection, wastewater COD was analyzed and diluted with tap water in order to achieve concentrations around 25, 50, 75, 100, 150 and 200 mg COD/L, which are in the range of influent/effluent concentrations reported for real scale HSSF CWs (Puigagut et al., 2007). Once diluted, wastewater was frozen until it was used in order to prevent any biological activity and consequent degradation of the organic matter.

During the experiment the CW-MFCs (2 graphite and 2 gravel replicates) were fed with increasing COD concentrations around every 24 hours and the electrical response under each experimental condition was recorded. The initial concentration was 0 mg/L (only tap water), in order to analyze the effect of the previously retained solids within the system on the electrical signal and served as a control. Wastewater for each concentration was defrozen the night before utilization. Samples were taken from the inlet wastewater and from the anodic chamber after ca. 24 hours of feeding. The experiment was replicated 4 times during 1.5 months (E1 to E4).

6.1.2.5   Analytical methods and calculations

Samples taken were stored at 4 °C before their analysis. Soluble and total COD were analyzed in triplicate according to Standard Methods (APHA, 2005). Conductivity and pH of the inlet wastewater was monitored during the E4 to detect any influence on system's performance (Endress+Hauser CLM381 and CRISON pH/mV 506). Electrical parameters including coulombic efficiencies were calculated on the basis of total COD according to (Logan et al., 2006).

6.1.2.6   *COD assessment in pilot-scale systems*

With the purpose of COD assessment in pilot-scale systems, 4 gravel-based lab-scale CW-MFCs as the ones described in section 6.1.2.1 were implemented in a pilot-scale constructed wetland treatment plant that is described in detail in Chapter 4.3. To test a wider range of COD concentrations, 2 of the lab-scale systems were fed with the pilot system's influent wastewater (real urban wastewater primary treated with a HUSB reactor), while the other 2 were fed with CWs effluent. While the pilot-scale CW unit was continuously fed, CW-MFCs implemented were semi-continuously fed. More into detail, wastewater within the lab-scale CW-MFCs was replaced 4 times per day (every 6 hours) with the influent/effluent wastewater of the pilot-scale CW unit. COD concentration of the CW-MFCs feeding wastewater was analyzed along 3 weeks two times per week at 12:00 in triplicates following Standard Methods (APHA, 2005). Voltages generated by CW-MFCs were continuously recorded every 5 minutes by means of a datalogger (Campbell Scientific CR1000).

6.1.3   Results

6.1.3.1   *Abiotic reactions assessment*

Bioindication accuracy depends on to what extent a relationship can be established between the current generated and the quantity of organic matter present in the media. In MFCs current is described to mainly derive from the organic oxidation conducted by the exoelectrogenic bacteria community established within the anodic biofilm. However, although being less efficient, abiotic oxidation reactions can also occur within the anodic chamber thus contributing to the overall current production (Lovley, 2006). These abiotically oxidized compounds can be both organic and inorganic (Lovley, 2006). Therefore, if other non-organic compounds that are not quantified in the COD analysis are oxidized using the anode as the electron acceptor, the reliability of the biosensing capacity can be altered. Urban wastewater is a very diverse substrate (Metcalf and Eddy, 1991) containing numerous different compounds, such as sulfides, that could abiotically react with the electrode generating electricity (Lovley, 2006; Ryckelynck et al.,

2005). Therefore, we assessed the contribution of non-biological reactions to the overall current production.

Results of this experiment are presented in Figure 6.1.2. As it can be seen, the number of electrons transferred during 48 hours was statistically higher in BIO_MFCs ($p<0.05$). Actually, the electrons transferred in ABIO_MFCs accounted for less than 2% of the electrons transferred in biological systems regardless the anodic material considered. Accordingly, it can be concluded that abiotic reactions did not contribute significantly to the electrical current and therefore they could not negatively affect biosensing accuracy.
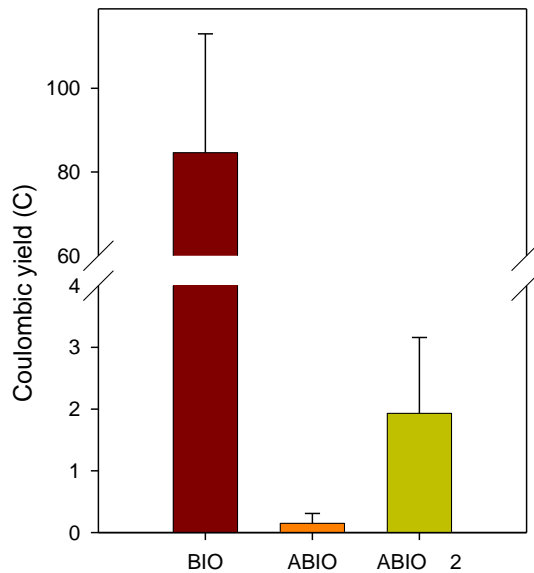
Figure 6.1.2 Coulombic yield

6.1.3.2 Cathode limitation assessment

Voltage generated by MFCs is always diminished by overpotentials and ohmic losses which can occur both in the anodic and the cathodic compartments (Logan et al., 2006). Cathodic overpotentials are main contributors to overall performance losses in MFCs (Rismani-Yazdi et al.,

2008) due to the slow kinetics of the oxygen reduction at graphite electrodes (Gil et al., 2003). Since the goal of the presented research was to test the electrical generation as function of the organic load introduced in the anodic chamber it was crucial to ensure that the cathode was not limiting the system's performance (Chang et al., 2004). One of the strategies to lower cathodic overpotentials is increasing the electrode surface area (Logan et al., 2006). Accordingly experimental trials were carried out to determine the cathode to anode ratio (C:A) that does not limit the system thus maximizing the voltage generated. Results are presented in Figure 6.1.3 and, as it can be seen, voltage increased concurrent with the cathodic area until the C/A ratio of 3.2 cm^2/cm^2. From then on, although the ratio was further increased up to 4 cm^2/cm^2, no statistical differences were found between voltages generated (Figure 6.1.3). Similar results were reported for conventional MFCs fed with acetate as carbon and electron source (Oh and Logan, 2006). Results obtained in this experiment suggested that, when wastewater was used as substrate, the ratio 3.2 cm^2/cm^2 was enough to avoid cathodic limitation. According to this result and considering that the optimal ratio for graphite based MFCs has been previously set to be 4:1 (Corbella et al., 2015a), the latter was the ratio implemented for the bioindication experiment.
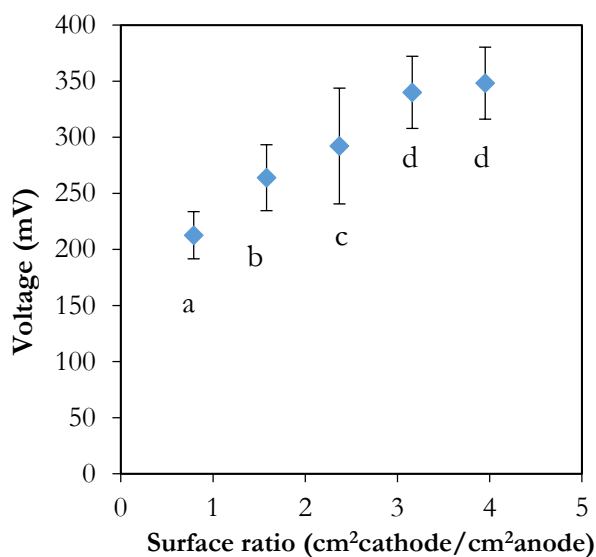
Figure 6.1.3 Voltage generated per cathode area. Note: letters indicate statistical differences

6.1.3.3 Conductivity effect

Solution conductivity is also one of the factors contributing to ohmic losses (Logan, 2008) and thus an influencing parameter on MFC voltage generation. Actually, one of the drawbacks of sustainable wastewater treatment using MFCs is the low solution conductivity of wastewaters (Liu and Cheng, 2014). Due to wastewater dilution with tap water, conductivity was altered leading to different conductivities along the experiment. Therefore, during E4 wastewater conductivity was measured in order to assess the effect of solution conductivity on MFC voltage generation. As seen in Figure 6.1.4 there was a clear relationship between conductivity and COD (both total and soluble) indicating that wastewater dilution affected the conductivity of the sample. In accordance with this result, Peixoto et al. (2011) also reported the clear relationship between power and conductivity. Furthermore, Peixoto et al. (2011) highlighted that the effect of conductivity was more pronounced between 1.1 ± 0.012 and 2.1 ± 0.012 mS/cm which matches our experimental conditions.

The positive relationship between both parameters (Figure 6.1.4) can question whether MFCs bioindicate solution conductivity rather than OM concentration of the media. Accordingly, there are two different solutions reported in the bibliography to avoid the effect of conductivity in wastewater MFC biosensors. Di Lorenzo et al. (2009) added a phosphate buffer (5M, pH7) to keep the wastewater conductivity constant. Otherwise, Peixoto et al. (2011) suggested the application of correction factors for measurements done under different environmental conditions (temperature, conductivity or pH). However wastewater is a very variable substrate and if biosensor MFCs are to be implemented in wastewater treatment plants, influent wastewater can vary hourly (Metcalf and Eddy, 1991). Consequently, both the addition of phosphate buffers and the application of correction factors would largely increase the complexity of MFC bioindication under real wastewater treatment conditions. Also a positive correlation between conductivity and COD has been reported from the continuous analysis of a WWTP influent wastewater (Daal-rombouts et al., 2013). This positive relationship between the two parameters in real urban wastewater can thus be considered a positive influence regarding bioindication purposes. Therefore, authors decided not to

correct the conductivity factor to simulate the real implementation of MFCs as biosensors in CW wastewater treatment plants.
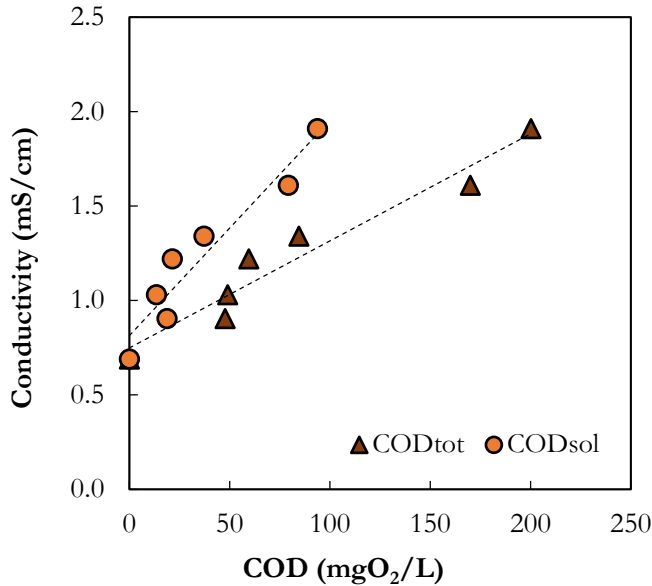
Figure 6.1.4 Linear relationship between total (triangles) and soluble (circles) chemical oxygen demand (COD) from E4 feeding wastewater. Note: R-squared values were 0.94 (total COD) and 0.92 (soluble COD)

6.1.3.4 Voltage patterns and COD removal

Voltages generated after the addition of wastewater within the systems followed a constant pattern along the entire experiment both for graphite and gravel MFCs. This representative pattern, which is presented in Figure 6.1.5, is also described for single-batch operation conventional MFCs elsewhere (Peixoto et al., 2011). The figure shows that a drastic increase of voltage was generated immediately after the addition of wastewater. Then voltages increased smoothly and continuously until the end of the experiment (after ca. 24h). When the system was emptied a steep decrease of voltages to zero was recorded. There was no difference between materials in terms of the voltage patterns, however, due to the higher conductivity of the anodic

material used, graphite MFCs generated higher voltages and intensities. More precisely, after 20h of contact time GRAPH-MFCs produced intensities of 163±92% higher than GRAVEL-MFCs. However, as it is further discussed, the bioindication capacity of the gravel systems was not compromised because of the lower intensities produced.

Removal efficiencies were calculated at 24 hours of contact time for all the concentrations tested and for the 4 experiments conducted (E1 to E4). Removal efficiencies increased with inlet COD concentrations (results not shown). Accordingly, regardless the experiment (E1 to E4) and the material considered, removal efficiencies obtained when the highest inlet concentrations were tested (204±52 mgCOD/L) averaged 56±9%. These rates are in the lower range of real scale HFCW removal efficiencies (Puigagut et al., 2007).
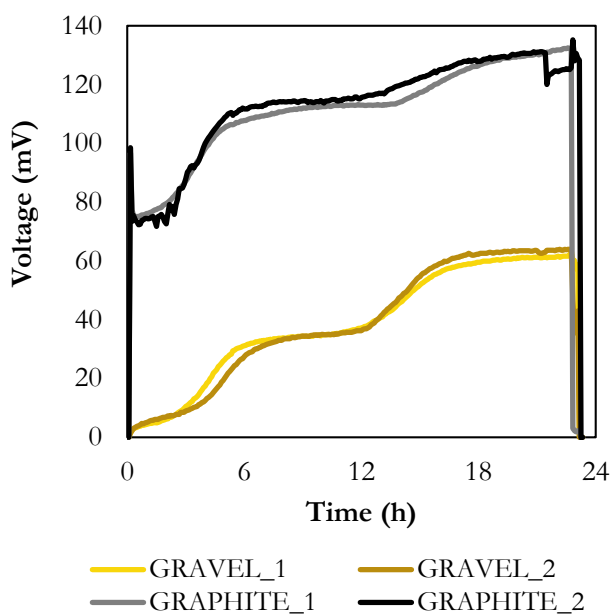
Figure 6.1.5 Voltage generated at 12.2 mg O_2/L of inlet COD concentration (E1)

6.1.3.5 Effect of COD concentration and contact time

A positive linear relationship could be established between current generated and inlet COD concentration after 20h of contact time. This linearity was found for both gravel and graphite MFCs. The determined slopes of the linear functions were also similar regardless the material considered (1156 and 1445 $mgO_2 \cdot L^{-1} \cdot mA^{-1}$, for gravel and graphite based MFCs respectively). However, as it has been previously described, the intensities recorded under the same organic matter concentrations were material dependent.

GRAPH-MFCs showed a better biosensing capacity both in terms of correlation coefficients (R-squared) obtained, which were found to be higher than in gravel systems (see Figure 6.1.6; 0.9 for GRAV-MFCs and 0.95 for GRAPH-MFCs) and also in terms of their bioindication range. As it can be seen in Figure 6.1.6, in GRAPH_MFCs, the linear relationship could be established between ca. 40 and 190 mg COD/L while the lower detection limit of GRAV_MFCs was 70 mg/L COD/L. Several authors used conventional MFCs as biosensing systems and found relationships between inlet organic matter concentrations (either by means of analyzing BOD or COD) and electric output (different electric parameters have been used to calibrate sensors: current, voltage, charge, etc.). The results obtained in this study are in accordance to the bibliography, where mostly linear relationships were described, always with R-squared values above 0.9 and similar bioindication ranges reported (Di Lorenzo et al., 2009; Gonzalez del Campo et al., 2013; Kim et al., 2003a; Peixoto et al., 2011). More precisely, Di Lorenzo et al. (2009), using a conventional air-cathode MFCs, found linear relationships between COD content of both real and synthetic wastewater (in the range of 40 to 200 mgO_2/L) and current generated.
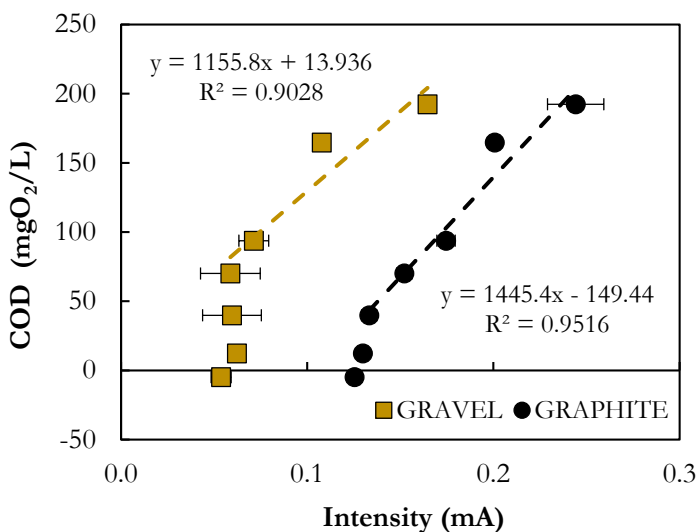
Figure 6.1.6 Linear relationship established between the intensity generated and the obtained COD for gravel and graphite CW-MFCs (data from E1 after 20h of contact time)

Response time is a crucial parameter for biosensing tools, especially for real-time monitoring (Liu and Mattiasson, 2002). During the first two experiments (E1 and E2), systems tested showed better R-squared values with increasing contact time. This trend was found for both the gravel and graphite systems. The longer the organic matter remains within the system, the more organic matter is degraded by means of exoelectrogenic pathways thus leading to a more significant relation between the quantity of organic matter and the electricity generated. Accordingly, our biosensors showed correlations with R-squared values higher than 0.8 for contact times higher than 10 hours regardless the material considered (Figure 6.1.7). However, the best results were obtained after 20h of contact time (Figure 6.1.6). The contact time appeared to be especially important in case of low organic matter concentrations. While at a contact time of 5h, both gravel and graphite system´s bioindication range was from 95 to 190 mgO$_2$/L, at 20h the lower detection limit decreased to 70 mgO$_2$/L in GRAV-MFCs and to 40 mgO$_2$/L in GRAPH_MFCs. These results indicate that the contact time had also an influence on MFC's bioindication range. This could be clearly seen in the first experiment in which the lower detection limit decreased with the increase of

the contact time (Figure 6.1.8). However, it is worth mentioning that graphite systems showed the capacity to bioindicate concentrations over 100 mgO$_2$/L starting from 1h of contact time.

However, the time needed to reach a linear relationship between parameters using real wastewater was significantly higher than those reported in literature when using glucose as MFCs carbon source. For example, Chang et al. (2004) reported response times of 60 min when using conventional MFCs fed with glucose and glutamic acid, while Kumlanghan et al. (2007), achieved response times between 3 and 5 minutes when using glucose as the carbon source. However, wastewater is composed of complex carbohydrates that need to be hydrolyzed to volatile fatty acids before being utilized by exoelectrogenic bacteria (Kiely et al., 2011) and therefore, the response time of systems fed with wastewater is significantly higher. If results obtained in this article are compared to MFC biosensors working with real wastewater they are within the range of results in literature. Peixoto et al. (2011) reports response times of about 10h for concentrations higher than 78±8 mgO$_2$/L. Also Di Lorenzo et al. (2009) reports response times of 13.75h.
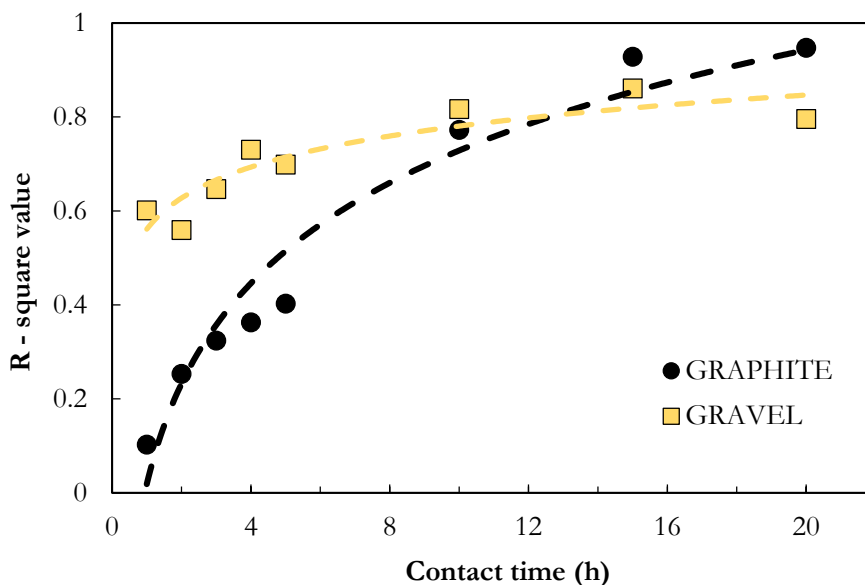
Figure 6.1.7 R-squared values obtained from the linear relationships established between the current generated and the COD tested as function of the contact time (h) during E1

However, unexpectedly, if we focus on the performance of the system when operated at low organic matter concentrations and low contact times results show an opposite trend. At low concentrations, higher inlet COD led to lower intensities recorded (Figure 6.1.8). This pattern was clearly accentuated during E3 and E4, regardless the material considered (results not shown). These results are evidence of the short operational stability of the CW-MFCs, at least when operated under the conditions here considered. As discussed below, the operation of the sensor with real wastewater drastically compromised its long-term stability. Contrary to our results, other studies reported long term stabilities using conventional MFCs. Accordingly, Di Lorenzo et al. (2009) reported 8 months of operation when feeding with artificial wastewater and Kim et al. (2003a), 5 years when feeding wastewater that was collected from a starch processing plant.
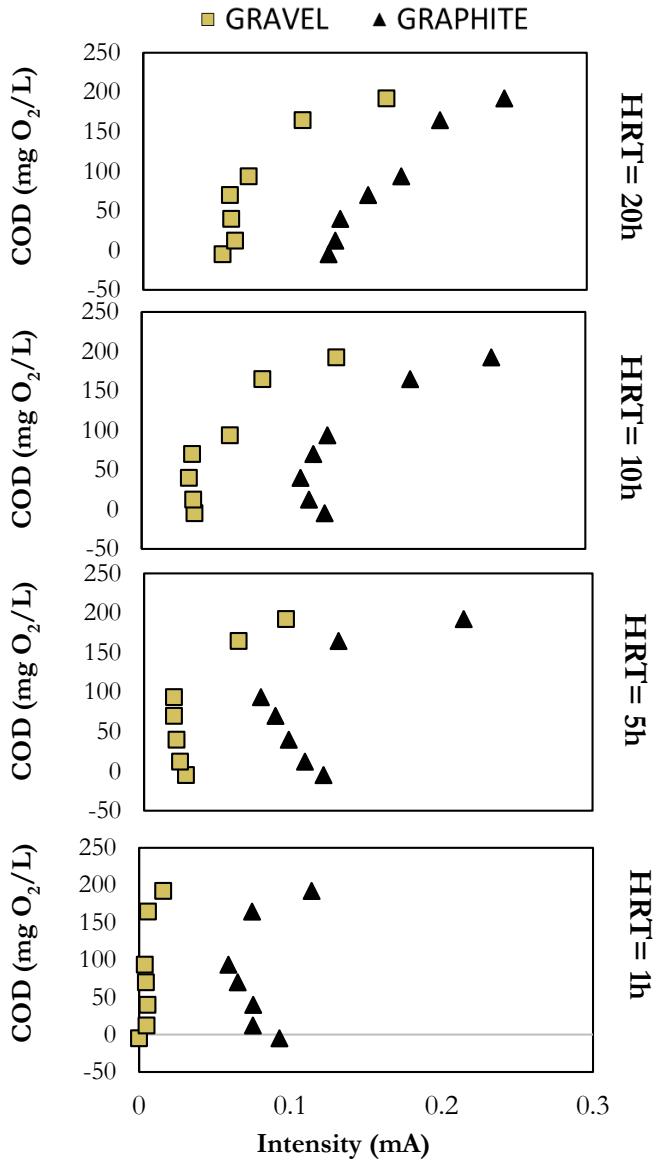
Figure 6.1.8 Intensities generated against COD tested at four different contact times (20, 10, 5 and 1h) during E1

6.1.3.6 Evolution of the biosensing capacity: effect of the accumulation of solids in the system

As a consequence of the retention of particulate organic matter, but also of the biofilm generation, the gravel matrix clogs over time (Kadlec and Wallace, 2009) and this is dependent, among other factors, on accumulated organic loading. This organic matter accumulated within the anodic volume can further participate in the generation of electricity and therefore must be assessed in terms of its effect on bioindication. In order to evaluate this effect, at the beginning of each experiment (E1 to E4), systems were fed with tap water (0 mg COD/L) and their electrical response was monitored. As can be seen in Figure 6.1.9, both in gravel and graphite MFCs, the intensity generated when tap water was introduced in the system, increased with the accumulated organic loading. Therefore, it was demonstrated that the organic matter used by exoelectrogens to generate electricity did not only come from the inlet wastewater, but also from the organic matter already present within the anodic volume. Also endogenous respiration could contribute to the generation of electricity during those periods (Chang et al., 2004). However, after the initial increase, a plateau was reached indicating that there was probably a limitation to the intensity generation. Clog matter composition in HSSF CWs has been studied and determined to be both of organic and inorganic nature (Knowles et al., 2011). Pedescoll et al. (2013a) reported the ratio of volatile solids (VS) with respect to TS to be always less than 50% regardless the operational conditions considered and Caselles-Osorio et al. (2007) determined VS/TS ratios between 10 and 20%. Accordingly, the accumulation of inorganic solids within the filter media could lead to a limitation of the transfer rates to the electrode to the point that the electrical response also decreases (Kim et al., 2003b). At that point, exoelectrogenic communities may have difficulties accessing the substrate due to the accumulation of non-biodegradable solids at the vicinity of the electrode, and may start a process of cell decay near the electrode leading to the decrease in the electricity generated. As a consequence, the system loses its capacity to bioindicate the quantity of organic matter present in inlet wastewater. Results corresponding to this decreasing phase are in accordance to those presented by Corbella et al. (2016a), who studied the possibility to bioindicate clogging by means of CW-

MFCs. In the study, the amount of electrons transferred to the system also decreased with the accumulation of sludge within the anode.

As it can be seen in Figure 6.1.9, bioelectrochemical response of MFCs in terms of current production was highly affected by the cumulated organic matter, leading to the necessity of frequent system recalibration in order to reestablish their short operational stability (Gonzalez del Campo et al., 2013; Kim et al., 2003b).
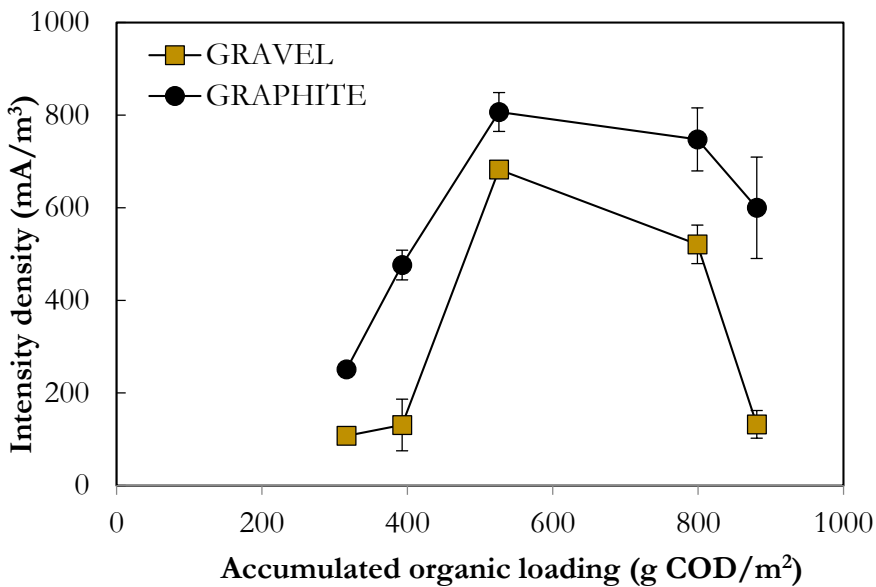
Figure 6.1.9 Intensity associated to tap water along the experimental period at 20h of contact time

6.1.3.7   *COD assessment in pilot-scale systems*

As it is shown in Figure 6.1.10, no linear relationship could be stablished between COD concentrations of the feeding wastewater and the voltage generated by CW-MFCs. However, two marked groups are drawn in the figure related to the voltages generated by CW-MFCs fed with influent wastewater, which had higher COD concentrations and therefore, generated higher voltages, and by those cells fed with the effluent wastewater of the CW

unit. The latter received wastewater with lower COD concentrations and, as a consequence, generated lower voltages. Therefore, this results suggest that although CW-MFCs may not be used as a direct substitute of the COD analysis, other alternatives could be considered such as its utilization as a range or qualitative bioindication tool. Looking at this results it is clear that when implemented before and after a pilot-scale CW unit and operated in a semi-continuous mode during 3 weeks, CW-MFCs were able to give different electrical responses to different COD concentration ranges thus potentially constituting a semi-continuous online "alarm tool" for sudden increases of pollution in wastewater treatment plants.
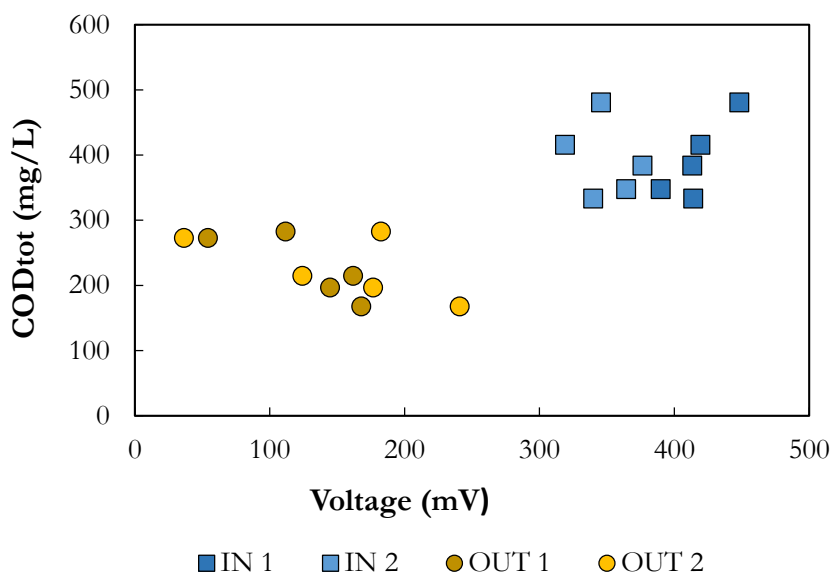
Figure 6.1.10 Voltage generated against total COD concentration of CW-MFC feeding wastewater (influent and effluent of the pilot-scale CW unit). Note: voltage was calculated as the average of the voltages generated during the 4 hours before and the 2 hours after the sampling time

6.1.4 Discussion

Microbial fuel cells are biolectrochemical systems that generate electricity using organic matter compounds present in wastewater as electron

donors. Bioindication accuracy depends on to what extend a relationship can be established between the current generated and the quantity of organic matter present in the media. However, the selection of wrong calibration parameters could negatively influence bioindication capacity. There are several organic matter indicators and electric output parameters which can be chosen to calibrate biosensors. In terms of the electrical output, voltage or current are just instant parameters, thus influenced by the dynamics of the oxidation processes, and are not necessarily representative of the total amount of OM present in the media. This can be confirmed in Figure 6.1.8, where it is shown that the voltage produced is significantly different depending on the contact time considered and actually, at least 10h of contact time is needed to get a acceptable bioindicating correlation. Accordingly, cumulative electric parameters, such as the coulombic yield may be considered (Gil et al., 2003; Kim et al., 2003b).

There is also the necessity to deeply discuss not only the ideal electric output calibration parameter, but also the chosen organic matter indicator. Organic matter concentration can be quantified with various parameters including soluble and total COD, BOD, TOC or volatile solids (APHA, 2005). Exoelectrogens have been described to oxidize several organic matter compounds (Thygesen et al., 2009), however organic matter present in wastewater is formed by very complex compounds which must be hydrolyzed before exoelectrogenic microorganisms can oxidize them (Kiely et al., 2011). Total COD, which is the calibration parameter used in this study, includes both biological and chemical oxygen demand and both from particulate and soluble compounds. Therefore, there are non-organic and particulate compounds which are accounted within the COD analysis that are not biologically degradable or at least, not during the biosensor's contact time. Therefore, a MFC based biosensor would never bioindicate this fraction, a fact that reduces its precision. Hence, a deep reflection is necessary concerning the organic matter indicator that best fits MFC bioindication characteristics.

Furthermore, Microbial fuel cells working with wastewater and, especially, in the context of constructed wetlands, are subjected to highly variable conditions which could reduce or even hinder MFCs bioindication capacity. Real wastewater has a very diverse composition and therefore,

organic matter in constructed wetlands can be degraded by means of several oxidation pathways. Accordingly, microbiological communities in constructed wetlands fed with real wastewater are also very diverse (Faulwetter et al., 2009) and vary along their lifespan as function of the environmental conditions to which they are subjected (Samsó and García, 2014). This variability of biofilms can result in a variation of the electrical response of the system and the consequent reduction of the sensor´s reproductivity. Although in MFC lab-scale studies microbiological preconditioning of the electrodes has been used in order to force the colonization of a certain community, the performance of MFCs operated with diluted digestate was found not to be influenced by the precolonization (Di Domenico et al., 2015). Furthermore, prevalence of a single type of organism in the biofilm would implicate very low substrate versatility. The operation of MFCs with real wastewater is also subjected to the introduction of particulate solids, which clog the anodic compartment with organic matter (Corbella et al., 2016a). As it has been demonstrated in this work, MFC-based biosensors operated with real wastewater generate a background level current which comes both from the oxidation of the accumulated solids within the system and through endogenous metabolism. This current is unassociated to the influent COD and varies over time which also hinders the sensor's precision and reproductivity. Therefore, longer studies should be developed to determine the required calibration frequency for such biosensing tools.

Finally, due to its diverse composition there are many compounds in wastewater that can be used as electron acceptors, like nitrates or sulfates, instead of the sensor's electrode (Liu and Logan, 2004). Coulombic efficiencies (CE) reported for wastewater MFC-biosensors are always below 26% (Di Lorenzo et al., 2009; He et al., 2005; Min et al., 2005; Wang et al., 2014). In accordance with bibliography, CE obtained in our experiments were always below 30% and 15% for graphite and gravel MFCs respectively. Moreover, CE was always below 10% and 5% for graphite and gravel MFCs, respectively, when inlet COD concentrations were higher than 50 mg O_2/L. These results indicate that most of the organic matter was actually removed by means of conventional anaerobic pathways such as methanogenesis or sulfate-reduction. These results could alter the measurement due to the fact that electrons are consumed within the anodic volume but not accounted in

the electrical signal. Although in MFC laboratory experiments, respiratory inhibitors can be used in order to avoid the effect of alternative electron acceptors (Chang et al., 2005), in the context of natural-based wastewater treatment technologies such as constructed wetlands, the introduction of any kind of reactant is not viable. On the contrary, electricity can also be generated through direct anodic oxidation of non-organic compounds, which could also hinder the bioindication capacity of the sensors. The most widely described process of this kind is direct sulfide oxidation (Lovley, 2006). In this case, electrons are transferred to the anode generating electricity that does not come from the oxidation of organic compounds. Although results presented in this study determined that abiotic reactions were not a significant part of the electricity generation, other configurations using different media must consider their potential effect. Under these conditions, MFC based sensors like the one proposed in this article, would only give a reliably signal in terms of bioindication if the composition of wastewater (including each of its specific compounds) would be proportional to the exoelectrogenically degradable OM concentration.

Although results obtained with this work indicate that linear relationships can be established between inlet COD and current generated, there are several factors that can affect the precision, the repeatability and the operational stability of the devices. Therefore, other alternatives should be considered when CW-MFCs are used as biosensing tools. These alternatives should include the utilization of the tool to determine the COD concentration range, instead of a precise COD value or, using it as a qualitative assessment device which alerts about the presence of particularly high organic matter concentration within the media.

6.1.5 Conclusions

Positive linear relationships could be established between current generated and inlet COD concentration. This linearity was found both for gravel and graphite MFCs. Although, the best results were obtained after 20h of contact time, our biosensors showed correlations with R-squared values higher than 0.8 also for contact times higher than 10 hours, regardless the material used.

Graphite based MFCs showed a better biosensing capactity than gravel systems in terms of the correlation coefficients (R-squared) obtained (0.9 for GRAV_MFCs and 0.95 for GRAPH_MFCs at 20h of contact time). Also in terms of the bioindication range graphite based MFCs showed better performances (bioindication range was between ca. 40 and 190 mg COD/L and between of 70 mg/L to 190 mg/L for graphite and gravel MFCs, respectively).

When comparing the electrons transferred to the external circuit in the abiotic CW-MFC to the ones transferred in the biological MFC, the abiotic current accounted for less than 2% of biological current regardless the anodic material considered.

Both in gravel and graphite MFCs, the intensity generated when tap water was introduced in the system (0 mg COD/L), increased with the accumulated organic loading. These results indicate that organic matter used by exoelectrogens to generate electricity did not only come from inlet wastewater, but also from organic matter already present within the anodic volume or from endogenous respiration. Authors suggest that further studies should be done in order to determine the recalibration or cleaning frequency to improve MFC's short operational stability.

Microbial fuel cells fed with wastewater and, especially, in the context of constructed wetlands, are subjected to very variable conditions which could hinder MFCs bioindication capacity. Although results obtained in this work indicate that linear relationships can be established between inlet COD and current generated, there are several factors that can have an effect on the precision, repeatability and operational stability of the devices. Therefore, other alternatives such as its utilization as qualitative assessment tools, should be considered for biosensor CW-MFCs.

CW-MFC used as a semi-continuous COD assessment tool in pilot plant experiments showed that CW-MFC are probably better suited as a qualitative, range-like assessment tool rather than a specific-value assessment tool.

6.2
Microbial fuel cells for clogging assessment in constructed wetlands

Abstract

Clogging in HSSF CW may result in a reduction of system's life-span or treatment efficiency. Current available techniques to assess the degree of clogging in HSSF CW are time consuming and cannot be applied on a continuous basis. Main objective of this work was to assess the potential applicability of microbial fuel cells for continuous clogging assessment in HSSF CW. To this aim, two replicates of a membrane-less microbial fuel cell (MFC) were built up and operated under laboratory conditions for five weeks. The MFC anode was gravel-based to simulate the filter media of HSSF CW. MFC were weekly loaded with sludge that had been accumulating for several years in a pilot HSSF CW treating domestic wastewater. Sludge loading ranged from ca. 20 kg TS·m^{-3} CW·year^{-1} at the beginning of the study period up to ca. 250 kg TS·m^{-3} CW·year^{-1} at the end of the study period. Sludge loading applied resulted in sludge accumulated within the MFC equivalent to a clogging degree ranging from 0.2 years (ca. 0.5 kg TS/m$^3_{wetland}$) to ca. 5 years (ca. 10 kg TS/m$^3_{wetland}$). Results showed that the electric charge was negatively correlated to the amount of sludge accumulated (degree of clogging). Electron transference (expressed as electric charge) almost ceased when accumulated sludge within the MFC was equivalent to ca. 5 years of clogging (ca. 10 kg TS/m$^3_{wetland}$). This result suggests that, although longer study periods under more realistic conditions should be conducted in the future, HSSF CW operated as a MFC has great potential for clogging assessment.

6.2.1 Introduction

Horizontal subsurface flow constructed wetlands (HSSF CW) are natural wastewater treatment systems that consist of gravel beds planted with macrophytes (García et al., 2010). In HSSF CWs pollutants are removed by means of physical, chemical and biological processes. HSSF CWs are generally used as secondary treatment units for the removal of contaminants (mainly organic matter) contained in domestic wastewater (Vymazal, 2002). HSSF CWs suffers from a progressive media obstruction, the so-called clogging process (Nivala et al., 2012). Clogging is a complex phenomenon derived from the retention of organic/inorganic particles, precipitation, biofilm formation and root-system growth (Knowles et al., 2011; Pedescoll et al., 2011a). It is widely accepted that clogging is one of the major operational problems of HSSF CWs (Kadlec and Wallace, 2009; Knowles et al., 2011; Nivala et al., 2012). Loss of hydraulic conductivity, creation of preferential water-flows, ponding and reduction of both treatment efficiency and system's life-span are some of the consequences of clogging (Caselles-Osorio and García, 2006; Knowles et al., 2011; Nivala et al., 2012; Wallace and Knight, 2006). To mitigate/reverse the clogging, different strategies (both preventive and/or restorative) are currently envisaged (Nivala et al., 2012). The quantification of total solids entrapped within the filter media is one of the most widely used techniques/strategies to assess the degree of clogging of HSSF CW. However, this procedure is time consuming, results in methodological inaccuracies due to sample manipulation and cannot be applied on a continuous basis. A great extent of the particulate material retained within HSSF CW is of organic nature (Knowles et al., 2011; Pedescoll et al., 2011a).Therefore, microbial fuel cells (MFC) could be a suitable technology for clogging assessment. MFCs are bioelectrochemical devices that generate electricity from the degradation of organic matter by means of exoelectrogenic bacteria as catalysts (Logan, 2008). MFC implementation in HSSF CWs has been already carried out taking benefit of the natural redox gradient existing between the upper and the bottom part of the treatment bed (Corbella et al., 2015a, 2014). Furthermore, MFCs have been also described as a suitable technology for bioindication purposes. Accordingly, MFC can produce an electric current proportional to the amount of organic matter present in the system. So far, the electric current generated by a MFC has been successfully correlated to organic substrates

such as artificial wastewater and real domestic wastewater (Chang et al., 2004; Di Lorenzo et al., 2009; Gil et al., 2003; Juang et al., 2011; Liu et al., 2000). The main objective of the present work was to determine whether MFC could be of use to quantitatively assess the total amount of organic particles retained within the gravel matrix so as to have a non-disruptive, continuous clogging assessment tool. To the authors knowledge this is the first attempt to use MFC as a clogging assessment tool.

6.2.2 Materials and methods

6.2.2.1 Set-up configuration

For the purpose of this work two lab-scale membrane-less microbial fuel cells (MFC) were set up and operated for 5 weeks (Figure 6.2.1). Each MFC consisted of two chambers (the cathodic and anodic chambers). The anodic chamber consisted of a PVC cylinder of 9 cm diameter and 30 cm high filled up with gravel (D_{60}=7.3; Cu=0.8; porosity=40%) and wrapped in stainless steel mesh (marine grade 316 L, 5 mm spacing between wires and 1 mm wire diameter). The anode chamber of the MFC was designed to simulate a core of a shallow wetland gravel bed where the electrons derived from exoelectrogens organic matter oxidation would be transferred to the electron collector (stainless steel mesh). In order to ensure adequate mixing conditions, water inside the anode chamber was continuously recirculated by means of a peristaltic pump (Damova MP-3035-6M; Toshiba VF-nC3). The cathode chamber consisted of a PVC cylinder placed just above the anode chamber filled up with 5 pieces of graphite felt (Alfa Aesar, 1.12 cm thick, 99.9 %; metal basis) of 60 cm^2 each inter-connected using stainless steel wires (marine grade 316L). A layer of glass wool was placed between the anode and the cathode chamber to avoid any oxygen leaking from the cathode (Venkata Mohan et al., 2008). The anode and the cathode were externally connected with copper wires through an external resistance of 1000 ohms. MFCs were operated under MFC mode thus no external energy was provided. Finally, voltages generated were measured across the external resistance and stored every minute by means of a datalogger (Campbell Scientific CR1000).
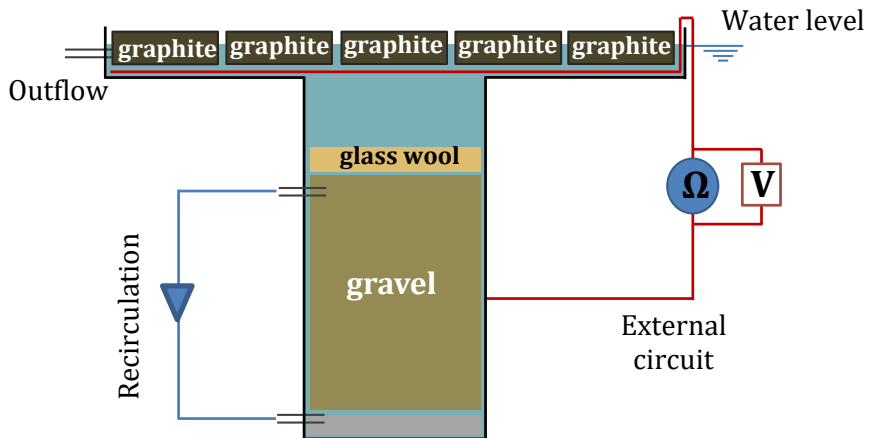
Figure 6.2.1 Outline of the MFC used to conduct the experiment

6.2.2.2 Sludge collection, experimental design and clogging assumptions

Sludge was supplied to the MFC on a weekly basis and current produced by the MFC was monitored for six days after each sludge load. MFCs were loaded in batch mode with sludge collected from the bottom of a HSSF CW pilot plant that had been treating domestic wastewater for several years (Corbella et al., 2015a). Solids accumulation in HSSF CWs is one of the main causes for clogging (Caselles-Osorio et al., 2007). Therefore, the introduction of HSSF CW sludge was considered to reasonably simulate the clogging process derived from solids accumulation within the filter media of HSSF CW. Note that although particles accumulation is one of the main processes contributing to the progressive media obstruction in wetlands, clogging is also the result of other processes such as precipitation, root growth, biofilm growth, etc. Therefore, it should be pointed out that in this work we are mainly addressing the clogging process derived from particles accumulation. The experiment lasted until the electric charge produced by the MFC was close to zero (five weeks). The sludge used to load the systems was collected and concentrated by sedimentation during 1 week at 4ºC. The concentrated sludge was then analyzed (CW sludge, Table 6.2.1) and later diluted with tap water to achieve the desired concentrations. Each week the

systems were fed with an increasing sludge load. Accordingly, sludge loading ranged from ca. 20 kg $TS \cdot m^{-3}$ $CW \cdot year^{-1}$ at the beginning of the study period up to ca. 250 kg $TS \cdot m^{-3}$ $CW \cdot year^{-1}$ at the end of the study period (Table 6.2.1). For the estimation of clogging time assayed we assumed that one year of clogging corresponds to the accumulation of 2 kg TS/m^3.year (Caselles-Osorio et al., 2007). The sludge provided to each of the MFC replicate was as homogeneously distributed as possible along the length of the anode. It is important to note that no wastewater was used in this experiment (sludge provided was mixed with tap water prior to supplying it to the MFC). Therefore, the electric signal recorded in our MFC derived solely from the oxidation of the sludge provided.

Cumulative sludge retention (C_w in kg TS/m^3) at a certain week (w) was calculated by means of the sum of the sludge accumulated during previous weeks (ACC_i in kg TS/m^3) plus the sludge loading at the week in course ($TS(IN)_w$ in kg TS/m^3)(Eq.1). The sludge accumulated (ACC_i) at a specific week (i) was calculated as the sludge supplied ($TS\ (IN)_i$ in kg TS/m^3) minus the sludge at the effluent ($TS(OUT)_i$ in kg TS/m^3) (Eq. 2).

$$C_w = \sum_{i=1}^{i=w-1} ACC_i + TS(IN)_w \quad (Eq.\,1)$$

$$ACC_i = TS(IN)_i - TS(OUT)_i \quad (Eq.\,2)$$

Table 6.2.1 Average and standard deviation (in brackets) of physical and chemical parameters of the sludge employed and experimental conditions tested

			TS (g/L)	VS (g/L)	DQO_{tot} (mgO₂/L)	DQO_{sol} (mgO₂/L)	NH_4^+ - N (mgNH₄⁺-N/L)	Sludge Loading (kgTS/m³CW·year)	Organic Loading (gO₂/m³CW·year)	Sludge accumulated (kgTS/m³CW)	Cumulative clogging time (years)
	Raw		39.1 (0.7)	25.5 (1.3)	58,100 (1,926)	933 (109)	87.4 (1.3)	-	-	-	-
Experimental	Weeks	1	1.0	0.6	741	87	2.8	21.5	15.5	0.5 (0.0)	0.2 (0.0)
		2	1.8	1.0	918	144	8.9	37.6	19.1	1.2 (0.1)	0.6 (0.1)
		3	4.0	2.5	1,640	249	10.7	82.5	34.2	2.9 (0.2)	1.4 (0.1)
		4	8.7	6.2	5,408	524	17.9	180.9	112.8	6.4 (0.8)	3.2 (0.4)
		5	12.1	8.5	16,700	674	24.6	252.0	348.3	10.2 (1.2)	5.1 (0.6)

6.2.2.3 Physico-chemical analyses

Samples were analyzed before and after each batch test (Table 6.2.1). Quality parameters analyzed during the experiment were total solids (TS), volatile solids (VS), total and soluble chemical oxygen demand (COD_{tot} and COD_{sol}) and ammonium nitrogen. Water quality parameters were analyzed following APHA (2005).

6.2.3 Results and discussion

6.2.3.1 MFC for clogging assessment

Clogging is one of the main operational problems of HSSF CWs and has been traditionally assessed by quantifying solids accumulated within the gravel matrix (Caselles-Osorio et al., 2007). However, this procedure is time consuming, cannot be applied on a continuous basis and results are not entirely reliable due to methodological inaccuracies derived from sample manipulation (Pedescoll et al., 2011a). To avoid direct measurements, two indirect clogging assessment methodologies have been proposed: (1) hydraulic conductivity measurements (Pedescoll et al., 2012, 2011c), and (2) tracer tests (Nivala et al., 2012). However, these methods still require exhaustive and time consuming sampling/measuring campaigns and cannot be applied on a continuous basis. In this study MFCs were loaded with increasing sludge dosages. As expected, the sludge retained within the systems had a direct effect on the MFC functioning (Figure 6.2.2). The electric current generated by a MFC has been positively correlated to the concentration of organic substrates such as artificial wastewater and real domestic wastewater (Chang et al., 2004; Di Lorenzo et al., 2009; Gil et al., 2003; Juang et al., 2011; Liu et al., 2000). However, our results showed that sludge retention was negatively correlated with both the electrons transferred to the circuit and the voltages generated for each batch test performed (Figure 6.2.2 and Figure 6.2.S1, respectively). Hence, the higher the sludge accumulated within the MFC the lower the amount of electrons transferred. These results are consistent with previous publications, where higher organic matter concentrations were negatively correlated to the current generated in a single chamber MFC (Sharma and Li, 2010). Author's hypothesis behind the reduction of current generated under higher particles accumulation is the bacterial activity decreases due to limited substrate availability. Accordingly, the lower the pore

space available within the filter media, the lower the substrate availability for exoelectrogens and the lower the amount of electrons transferred. This hypothesis is consistent with that described for the general functioning of bacterial activity in horizontal subsurface flow constructed wetlands (Samsó and García, 2014).

Correlations between specific electric charge and solids accumulated followed a potential equation, regardless the contact time considered (Figure 6.2.2). Results showed that after an equivalent clogging time of ca. 5 years the MFC was no longer able to produce any significant electric charge (Figure 6.2.2). This result suggests that, although longer study periods under more realistic conditions should be performed in the future, MFC has great potential to be used as an indirect, continuous clogging assessment tool in constructed wetlands.

It is important to point out that the specific electric charge (given a certain amount of sludge accumulated within the MFC) increased with the contact time (especially between 0.2 and 1.4 years of clogging). Accordingly, the longer the elapsed time for a given amount of sludge accumulated the higher the electric charge produced. The production of a higher electric charge after a longer elapsed time is consistent with the fact that hydrolytic bacteria shall cut up complex substrates (such as the sludge here employed) before the exoelectrogens can oxidize simple substrates (such as volatile fatty acids) and transfer the electrons to the anode (Cusick et al., 2010; Kiely et al., 2011). Overall, although the electric charge was dependent on the contact time, the level of correlation between the electric charge and the amount of sludge retained (or clogging time assayed) was of similar magnitude (always showing $R^2 > 0.9$), regardless the contact time considered (Figure 6.2.2). Therefore, looking at our results it seems that there is no specific contact time out of those here considered that results in better MFC performance in terms of clogging assessment.
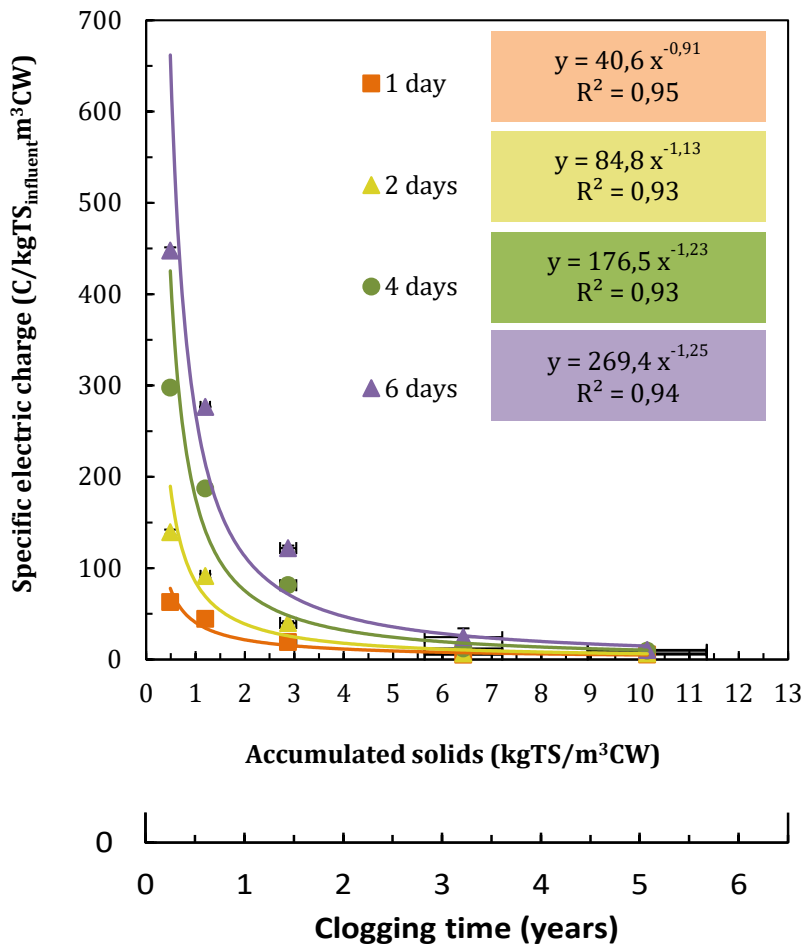
Figure 6.2.2 Specific electric charge against both the accumulated total solids (TS) and the equivalent clogging time for a selected set of days of contact time
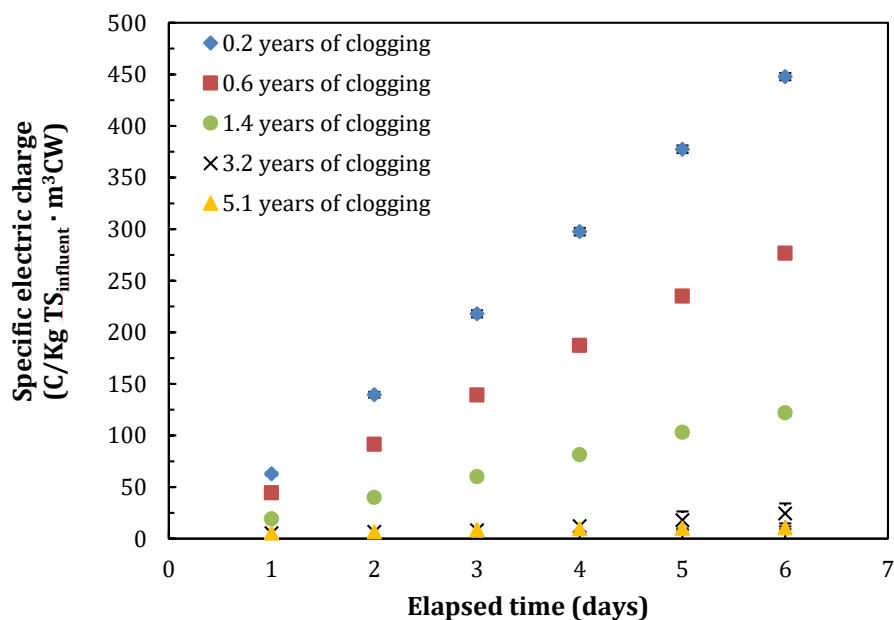
Figure 6.2.3 Specific electric charge as function of the elapsed time for a given estimated clogging time. Note that error bars are depicted within the figure, yet due to low data variability it is difficult to recognize them

6.2.4 Conclusions

The sludge retained within the microbial fuel cells (MFC) had a direct effect on the MFC performance. Accordingly, the higher the sludge accumulated within the MFC the lower the amount of electrons transferred. The electric charge decreased as function of sludge retained following a potential equation.

After ca. 5 years of estimated clogging time the MFC was no longer able to produce any significant electric charge. This result suggests that, although longer study periods under more realistic conditions should be performed in the future, MFC has great potential to be used as an indirect, continuous clogging assessment tool in constructed wetlands.

Electric charge was dependent on the contact time. Accordingly, the higher the contact time the higher the amount of electrons transferred to the circuit. However, the correlation between the electric charge and the amount

of sludge retained (or clogging time essayed) was of similar magnitude (always showing $R^2>0.9$), regardless the contact time considered. Therefore, the accuracy of the MFC for clogging assessment was independent of the contact time considered.

Life cycle and economic assessment of CW-MFCs

Abstract

The aim of this study was to assess the environmental impact of Microbial fuel cells (MFCs) implemented in constructed wetlands (CWs). To this aim a Life Cycle Assessment (LCA) was carried out comparing three scenarios: 1) a conventional CW system (without MFC implementation); 2) a CW system coupled with a gravel-based anode MFC, and 3) a CW system coupled with a graphite-based anode MFC. All systems served a population equivalent of 1,500 p.e. They were designed to meet the same effluent quality. Since MFCs implemented in CWs improve treatment efficiency, the CWs coupled with MFCs had lower specific area requirement compared to the conventional CW system. The functional unit was 1 m^3 of wastewater. The LCA was performed with the software *SimaPro® 8*, using the CML-IA baseline method. The three scenarios considered showed similar environmental performance in all the categories considered, with the exception of Abiotic Depletion Potential. In this impact category, the potential environmental impact of the CW system coupled with a gravel-based anode MFC was around 2 times higher than that generated by the conventional CW system and the CW system coupled with a graphite-based anode MFC. It was attributed to the large amount of less environmentally friendly materials (e.g. metals, graphite) for MFCs implementation, especially in the case of gravel-based anode MFCs. Therefore, the CW system coupled with graphite-based anode MFC appeared as the most environmentally friendly solution which can replace conventional CWs reducing system footprint by up to 20%. An economic assessment showed that this system was around 1.5 times more expensive than the conventional CW system.

7.1 Introduction

Horizontal subsurface flow constructed wetlands (HSSF CWs) are natural wastewater treatment systems in which pollutants are removed by means of physical, chemical and biological processes (García et al., 2010). They constitute an alternative to conventional systems for wastewater treatment (e.g. activated sludge systems) in small communities due to their low energy requirement and easy operation and maintenance (Puigagut et al., 2007b). Nevertheless, HSSF CWs are characterized by higher specific area requirement when compared to conventional technologies (2- 5 vs.<1 m^2 p.e.$^{-1}$, respectively). In order to overcome this drawback, several intensifying strategies (e.g. forced aeration) has been lately investigated (Austin and Nivala, 2009; Wu et al., 2014). However, these strategies often result in a significant increase in energy consumption when compared to conventional HSSF CW designs.

Microbial Fuel Cells (MFCs) are bioelectrochemical devices that generate electricity from organic matter by means of exoelectrogenic bacteria (Logan et al., 2006). These bacteria oxidize organic compounds and transfer the resulting electrons to an electrode (anode). From the anode, electrons flow through an external circuit (containing a resistor) to the cathode, where they are used to reduce an electron acceptor (e.g. oxygen) (Rabaey and Verstraete 2005). Therefore, MFCs performance depends on the redox gradient between electrodes (anode and cathode).

The presence of organic matter in wastewater and the naturally generated redox gradient between the upper layer (in aerobic conditions) and the deeper layers (in anaerobic conditions) of HSSF CW treatment bed, are favourable conditions for the implementation of MFCs in CW systems (Corbella et al., 2014; García et al., 2003). During the last decade, several studies have demonstrated the synergy between MFCs and HSSF CWs (Corbella et al., 2016b, 2015a). Indeed, the implementation of MFCs in HSSF CWs may lead to important benefits. First of all, it provides an energy surplus that can partially cover the energy input necessary for wastewater treatment (Corbella et al., 2015a). Moreover, MFCs can stimulate the degradation of organic matter present in wastewater by fostering more efficient degradation pathways carried out by exoelectrogenic bacteria (Katuri et al., 2011; Srivastava et al., 2015). As a consequence, the implementation of MFCs in

HSSF CWs can improve CWs treatment efficiency and reduce their surface requirement. However, materials used for conventional MFCs electrodes (e.g. carbon fiber, stainless steel) are expensive materials with poor environmental performance (Foley et al., 2010; Gude, 2016; Liu and Cheng, 2014; Zhou et al., 2011). Therefore, although energy inputs and surface area requirement could be reduced, both costs and environmental impacts could significantly increase when implementing MFCs in CW treatment systems.

Even if several studies which analyse the environmental impacts of CW systems have been carried out (Dixon et al., 2003; Fuchs et al., 2011; Machado et al., 2007; Yıldırım and Topkaya, 2012), there is still no study assessing environmental impacts of CW systems coupled with MFCs.

The objective of this study was to evaluate the environmental impacts caused by HSSF CWs coupled with MFCs made of different materials. To this aim a Life Cycle Assessment (LCA) was performed comparing three alternatives: i) a conventional CW system (without MFCs implementation); ii) an HSSF CW system coupled with a gravel-based anode MFC; iii) an HSSF CW system coupled with a graphite-based anode MFC.

An economic evaluation of the considered scenarios was also conducted.

7.2   Materials and methods

7.2.1   Constructed wetland systems design

The conventional CW system was a hypothetical wastewater treatment plant designed to serve a population equivalent of 1,500 p.e. and treat 292.5 m^3 of wastewater per day. It comprised a primary treatment (i.e. septic tank) followed by HSSF CWs. The CW unit consisted of 3 basins filled up with granitic gravel (D60=7.3; Cu=0.8; porosity=40%) and planted with *Phragmites australis* (Pedescoll et al., 2013b).

The CW unit was designed according to (García and Corzo, 2008). First of all, the total surface area was determined using the following expression:

$$S = \frac{Q}{k_A} \ln\left[\frac{C_0}{C_1}\right]$$
(Eq. 1)

Where

S= total CW surface, m^2

Q= inlet flow rate, $m^3\ d^{-1}$

k_A= first order rate constant for BOD removal, $m\ d^{-1}$

C_0= BOD inlet concentration, $mg\ L^{-1}$

C_1= BOD outlet concentration, $mg\ L^{-1}$

In this case, the first order rate constant for BOD removal (k_A) was considered to be 0.08 m d^{-1} (García and Corzo, 2008). Then, the hydraulic sizing was conducted by applying the Darcy's law and considering a porosity of 35%, a hydraulic conductivity of 5,000 $m^3\ m^{-2}\ d^{-1}$, a safety factor of 7, a slope of 0.01 m m^{-1}, a wetland depth of 0.35 m and a water depth of 0.3 m (García et al., 2005; García and Corzo, 2008).

The design of the CW systems coupled with gravel and graphite-based anode MFCs was carried out taking into account that the implementation of MFCs in CWs stimulates degradation processes leading to higher k_A values compared to conventional CWs (without MFCs) (Srivastava et al., 2015). In these cases, the k_A was estimated considering the results obtained in previous experiments conducted at the Universitat Politècnica de Catalunya-BarcelonaTech (UPC) (Barcelona, Spain). These experiments showed a decrease in outlet BOD concentrations as a consequence of the implementation of MFCs in lab-scale HSSF CWs, which indicates an increase of the BOD removal rate constant in CW systems coupled with MFCs (Corbella and Puigagut, in preparation). In accordance with the results of this study, the k_A was increased to 0.092 m d^{-1} and 0.098 m d^{-1} for the CW system coupled with gravel-based anode MFC and the CW system coupled with graphite-based anode MFC, respectively. It is important to note that since all CW systems here considered were designed to provide the same effluent quality (25 $mg_{BOD}\ L^{-1}$), higher k_A values resulted in lower specific area requirements (Eq. 1).

MFCs cathode was designed to be a 12 cm deep layer of crushed graphite placed at the upper part of the CW (in contact with the atmosphere) covering most of the surface of the gravel bed. This design was taken from the recommendations given elsewhere (Corbella et al., 2016b) to make sure that the cathode remains always in contact with the water table and the atmosphere. Furthermore, the anodic volume was determined according to the optimal cathode to anode ratio (4:1) as recommended by (Corbella et al., 2015a). MFCs anode was placed at a distance of 2 m from the inlet distribution zone (after the initial coarse gravel zone). The anode was considered to be made of gravel or crushed graphite (Figure 7.1). Even though gravel is not a conductive material, it has been reported that it provides a suitable surface for the establishment of exoelectrogenic communities if an electron collector (e.g. stainless steel mesh) is provided (Corbella et al., 2015a). Therefore, in gravel-based anode a stainless steel mesh (0.5 cm-mesh) was placed at every 5 cm depth along the whole anode surface. CW systems characteristics and design parameters are summarised in Table 7.1.

Table 7.1.A CW systems characteristics and design parameters

	Unit	Scenarios [a]		
		S1	**S2**	**S3**
System characteristics				
Inlet BOD concentration [b]	mg_{BOD} L^{-1}	168	168	168
Outlet BOD concentration [c]	mg_{BOD} L^{-1}	25	25	25
Flow rate	m^3 d^{-1}	292.5	292.5	292.5
Population equivalent	p.e.	1,500	1,500	1,500
BOD removal efficiency	%	85	85	85
Design parameters				
Hydraulic conductivity	m^3 m^{-2} d^{-1}	5,000	5,000	5,000
First order rate constant for BOD removal (k_A)	m d^{-1}	0.08	0.092	0.098
Organic Loading Rate (OLR)	g_{BOD} m^{-2} d^{-1}	6.00	6.90	7.40
Hydraulic Loading Rate (HLR)	m d^{-1}	0.036	0.041	0.044

[a] S1: conventional CW system (without MFC); S2: CW system coupled with a gravel-based anode MFC; S3: CW system coupled with a graphite-based anode MFC

[b] Influent concentration at the treatment plant was 240 mg BOD L^{-1}. Primary treatment was assumed to remove 30% of the BOD concentration.

[c] Discharge legislation limit (MAGRAMA, 2007)

Table 7.1.B CW systems characteristics and design parameters

		Scenarios [a]		
	Unit	**S1**	**S1**	**S1**
Constructed wetlands				
Number of constructed wetland cells	-	3	3	3
Constructed wetland cell dimensions	*m (D×L×W)*	$0.3 \times 60 \times 45.5$	$0.3 \times 52.5 \times 45.5$	$0.3 \times 49 \times 45.5$
Total surface area	m^2	8,190	7,166	6,688.5
Specific area requirement	$m^2 p.e.^{-1}$	5.46	4.78	4.46
Microbial Fuel Cells				
Anode — Material	-	-	gravel	graphite
Anode — Volume	m^3	-	64.23	59.59
Cathode — Material	-	-	graphite	graphite
Cathode — Volume	m^3	-	264.81	245.7

[a] S1: conventional CW system (without MFC); S2: CW system coupled with a gravel-based anode MFC; S3: CW system coupled with a graphite-based anode MFC

[b] Influent concentration at the treatment plant was 240 mg BOD L⁻¹. Primary treatment was supposed to remove 30% of the BOD concentration.

[c] Discharge legislation limit (MAGRAMA, 2007)
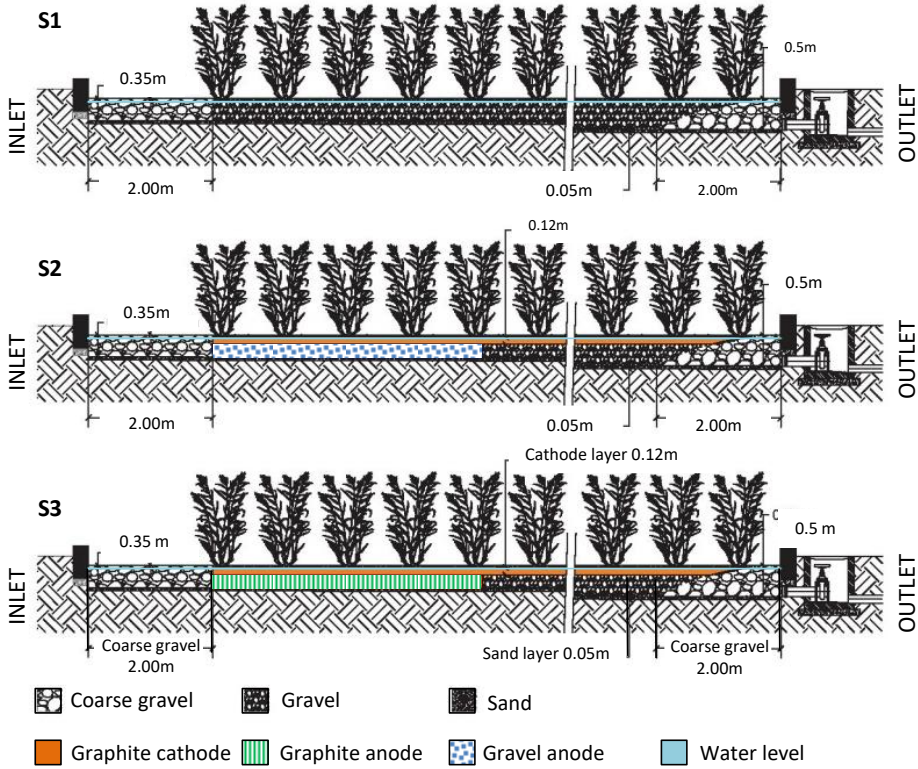
Figure 7.1 Schematic cross section of CWs for the considered scenarios. S1: conventional CW system (without MFC); S2: CW system coupled with a gravel-based anode MFC; S3: CW system coupled with a graphite-based anode MFC

7.2.2 Life Cycle Assessment

LCA is a standardized methodology for the evaluation of the potential environmental impacts generated by a product, process or service using a cradle to grave approach (ISO, 2000; ISO, 2006). It identifies and quantifies the environmental burdens associated with energy and materials used (inputs) and waste released into the environment (outputs) during the whole life cycle. LCA is mainly used to compare different competing products or technologies, as well as to identify improvement alternatives for a single product or technology. The methodological framework for LCA consists of the following phases: goal and scope definition; inventory analysis; impacts assessment and interpretation of the results (ISO, 2006). The following sections describe the specific contents of each phase.

7.2.3   *Goal and scope definition*

The goal of this study was to assess and compare the potential environmental impacts generated by HSSF CWs for wastewater treatment coupled with MFCs made of different materials. To this aim, the following scenarios were considered:

1) Conventional CW system (without MFC) (S1);

2) CW system coupled with a gravel-based anode MFC (S2);

3) CW system coupled with a graphite-based anode MFC (S3).

The functional unit was 1 m^3 of treated water.

The system boundaries included unit processes related to system construction and operation over a period of 20 years. Input flows associated with construction materials and energy resources (electricity) were comprehensively studied for all alternatives. Outputs consisted of direct greenhouse gas (GHG) emissions. The end-of-life of infrastructures and equipment were excluded from the scope of LCA, since it was considered to not significantly influence the overall impact (Lopsik, 2013; Machado et al., 2007). Sludge disposal was not accounted for, since its contribution only represents a minor fraction of the overall impact (Garfí and Ferrer, 2017). Transportation of construction materials was not considered. Their contribution to the overall impact can be neglected, since locally produced materials are supposed to be used (Fuchs et al., 2011). The effluent discharge was not included within the system boundaries, since the CW systems were designed in order to produce a same quality final effluent.

The system expansion method has been used to quantify the impacts generated by by-products, as suggested by ISO standard (ISO, 2006). It consists of considering the environmental benefits of recovered resources (by-products) by expanding the system boundaries to include the avoided impacts of conventional production. In this study, the avoided burdens of using electricity produced by MFCs instead of electricity supplied through the grid were considered.

7.2.4 *Inventory analysis*

The results of the inventory analysis for the three investigated CW systems are summarized in Table 7.2. Inventory data regarding construction processes, construction materials and electricity consumption were gathered from the construction projects performed in the frame of this study. CH_4 emissions from the conventional CW system were estimated considering the emissions rate found in previous studies carried out in a pilot plant of HSSF CWs implemented at the Universitat Politècnica de Catalunya-BarcelonaTech (UPC) (Barcelona, Spain) (Corbella and Puigagut, 2014). In order to estimate CH_4 emissions from the CW systems coupled with MFCs, the MFC efficiency in reducing CH_4 fluxes found by Rizzo et al. (2013) was considered. Regarding the N_2O emissions, the emission rate proposed by Mander et al. (2008) was taken into account for all scenarios. CO_2 emissions were not included in the inventory, since CO_2 from biogenic sources does not contribute to global warming potential (Doorn et al., 2006). Electricity produced by MFCs were determined considering the results obtained from lab-scale experiments carried out at the Universitat Politècnica de Catalunya-BarcelonaTech (UPC) (Barcelona, Spain) (Corbella and Puigagut, in preparation; Corbella and Puigagut, 2016b). All materials and energy inputs, as well as direct GHG emissions, were determined based on the functional unit. Background data were obtained from the *Ecoinvent 3.1* database (Moreno-Ruiz et al., 2014; Weidema et al., 2013). The Spanish electricity mix (i.e: natural gas 39%; nuclear 19%; coal 15.50%; wind 10.90%; hydro 8.80%; liquid fuels 5.80% and solid biomass 1%) was used for the electricity requirement.

Table 7.2 Wastewater treatment inventory for scenarios S1, S2 and S3. Values are referred to the functional unit (1 m³ of water)

	Units	Scenarios [a]		
		S1	**S2**	**S3**
Inputs				
Construction materials				
Inlet pumping station				
Concrete	$m^3 m^{-3}$	5.77E-06	5.77E-06	5.77E-06
Metals	$kg\ m^{-3}$	8.51E-04	8.51E-04	8.51E-04
Coating	$kg\ m^{-3}$	1.19E-04	1.19E-04	1.19E-04
Plastics	$kg\ m^{-3}$	4.41E-06	4.41E-06	4.41E-06
Septic tank				
Concrete	$m^3 m^{-3}$	3.37E-05	3.37E-05	3.37E-05
Metals	$kg\ m^{-3}$	3.32E-03	3.32E-03	3.32E-03
Coating	$kg\ m^{-3}$	6.23E-04	6.23E-04	6.23E-04
Plastics	$kg\ m^{-3}$	2.02E-05	2.02E-05	2.02E-05
Pumping stations				
Concrete	$m^3 m^{-3}$	6.47E-06	6.47E-06	6.47E-06
Metals	$kg\ m^{-3}$	9.70E-04	9.70E-04	9.70E-04
Coating	$kg\ m^{-3}$	1.21E-04	1.21E-04	1.21E-04
Plastics	$kg\ m^{-3}$	1.32E-05	1.32E-05	1.32E-05
Constructed wetlands and Microbial fuel cells				
Concrete	$m^3 m^{-3}$	1.75E-05	1.63E-05	1.57E-05
Metals	$kg\ m^{-3}$	8.42E-04	5.32E-03	7.71E-04
Coating	$kg\ m^{-3}$	1.19E-05	1.19E-05	1.19E-05
Plastics	$kg\ m^{-3}$	7.92E-03	7.01E-03	6.58E-03
Gravel and sand	$kg\ m^{-3}$	2.76E+00	1.83E+00	1.59E+00
Bricks	$kg\ m^{-3}$	3.86E-02	3.59E-02	3.46E-02
Graphite	$kg\ m^{-3}$	-	2.99E-01	3.44E-01
Storage tank				
Concrete	$m^3 m^{-3}$	5.69E-05	5.69E-05	5.69E-05
Metals	$kg\ m^{-3}$	5.31E-03	5.31E-03	5.31E-03
Coating	$kg\ m^{-3}$	5.82E-04	5.82E-04	5.82E-04
Plastics	$kg\ m^{-3}$	2.39E-06	2.39E-06	2.39E-06
Pipelines				
Plastics	$kg\ m^{-3}$	1.29E-04	1.29E-04	1.29E-04

		Scenarios [a]		
	Units	**S1**	**S2**	
Operation				
Electricity	*kWh m⁻³*	3.10E-01	3.10E-01	3.10E-01
Outputs				
Emissions to air (direct GHG emissions)				
CH₄	*g m⁻³*	10.89	8.49	8.49
N₂O	*g m⁻³*	0.01	0.01	0.01
Avoided products				
Electricity produced by MFCs	*kWh m⁻³*		1.44E-02	1.44E-02

[a] *S1: conventional CW system (without MFC); S2: CW system coupled with a gravel-based anode MFC; S3: CW system coupled with a graphite-based anode MFC*

7.2.5   Impact assessment

The LCA was performed using the software *SimaPro® 8* (Pre-sustainability, 2014). Potential environmental impacts were assessed by the CML-IA baseline method following the ISO standard procedure (ISO, 2000). The analysis focused on the following impact categories: Abiotic Depletion, Abiotic Depletion (fossil fuels), Global Warming Potential, Ozone Layer Depletion, Acidification, Eutrophication and Photochemical Oxidation. In this study only classification and characterisation phases were performed.

7.2.6   Sensitivity Analysis

A sensitivity analysis evaluates the influence of the most important assumptions have on the results. It consists of defining some scenarios, alternative to that assumed as a base case, and comparing the potential environmental impacts with those of the base case scenarios. To this end, selected parameters are changed into reasonable ranges of variation to check if the outcomes of the LCA can be heavily dependent on some of the assumptions. In this study, two parameters were evaluated (Table 7.3): i) the k_A in scenarios S2 and S3 (CW systems with gravel and graphite-based anode MFCs, respectively); and, ii) the electricity produced by MFCs in scenarios S2 and S3 (CW systems with gravel and graphite-based anode MFCs, respectively).

Regarding the k_A, two alternatives were considered: 0.138 and 0.162 m d^{-1}, which correspond to an increase of 50 and 75% with respect to the k_A taken into account in scenario 2 (0.092 m d^{-1}). These values have been chosen considering that MFCs in CWs can produce an improvement in treatment efficiencies higher than those used in the base case scenarios (Aguirre-Sierra et al., 2016). In order to carry out the sensitivity analysis, the CW systems in scenarios S2 and S3 were redesigned taking into account the above-mentioned k_A values. Since these k_A values were higher than those of the base case scenarios, the CW systems considered for the sensitivity analysis had higher treatment efficiency and lower specific area requirement compared to that of the base case scenarios (Table 7.3).

Concerning the electricity produced by MFCs, two alternatives were analysed: 40 Wh m^{-3} and 70 Wh m^{-3}. These values were chosen as they represent a middle and high energy production scenario for conventional MFC systems treating wastewater, respectively (Ge et al., 2014; Logan and Rabaey, 2013).

Table 7.3 Scenarios and parameters considered in the sensitivity analysis

Scenarios [a]	Microbial fuel cells		k_A	Electricity produced by MFCs	Specific area requirement
	Anode	**Cathode**	*m d^1*	*Wh m^3*	*m^2 p.e.$^{-1}$*
S1	**-**	-	0.080	-	5.42
S2 (base case)	Gravel	Graphite	0.092	14.4	4.74
S2A	Gravel	Graphite	0.138	14.4	3.14
S2B	Gravel	Graphite	0.162	14.4	2.68
S2C	Gravel	Graphite	0.092	40	4.74
S2D	Gravel	Graphite	0.092	70	4.74
S3 (base case)	Graphite	Graphite	0.098	14.4	4.42
S3A	Graphite	Graphite	0.138	14.4	3.14
S3B	Graphite	Graphite	0.162	14.4	2.68
S3C	Graphite	Graphite	0.098	40	4.42
S3D	Graphite	Graphite	0.098	70	4.42

7.2.7 *Economic assessment*

The economic analysis was conducted comparing the capital cost of the three CW systems (scenarios S1, S2 and S3). In addition, the scenarios with lower specific area requirement (scenarios S2A, S2B, S3A and S3B, Table 7.3) considered in the sensitivity analysis were also taken into account. In all scenarios, prices were provided by local companies. The capital cost included the cost for earthmoving, construction materials purchase and electrical works. For all scenarios, a lifespan of 20 years was considered.

CWs implemented with MFCs would probably require more material replacement than conventional CWs configurations. However, MFC implemented in CWs would reduce biomass growth within the filter media (Park and Zeikus, 2000), reducing clogging and its derived operation and maintenance costs. Overall, operation and maintenance costs were assumed to be the same in all scenarios and, thus, they were not included in the analysis.

7.3 Results and discussion

7.3.1 *Life Cycle Assessment*

The potential environmental impacts associated with each scenario are shown in Figure 7.2.

All the alternatives showed a similar environmental performance in all the categories analysed, with the exception of Abiotic Depletion Potential. In this impact category, the potential environmental impact of scenario S2 (CW system coupled with a gravel-based anode MFC) was around 2 times higher than that generated by scenarios S1 and S3 (conventional CW system and CW system coupled with a graphite-based anode MFC, respectively) (Figure 7.2). It was due to the fact that, despite the CW systems coupled with MFCs showed lower specific area requirement compared to the conventional CW system, they require a large amount of less environmentally friendly materials (i.e. metals and graphite) for MFCs implementation (Table 7.2). In particular, the high impact caused by the CW system coupled with a gravel-based anode MFC (scenario S2) in the Abiotic Depletion category was mainly attributed to the large amount of stainless steel required for the electron collector at the anode (stainless steel mesh) (Table 7.2). It was in accordance with previous studies which observed that the potential environmental impact of a CW

system would increase by around 30% of the overall impact if gravel and sand were replaced with less environmentally friendly materials (i.e. lightweight expanded clay aggregate) (Lopsik, 2013).

Since CW systems are extensive, low-tech and low energy technologies, their life cycle is mainly influenced by construction. For all scenarios, the contribution of the construction and operation stages in Abiotic Depletion impact category accounted for 88-95% and 5-12% of the total impact, respectively. It was in accordance with previous studies which assessed the environmental impacts of conventional CW systems (Dixon et al., 2003; Fuchs et al., 2011; Machado et al., 2007). With regards to Abiotic Depletion (fossil fuels), Acidification and Eutrophication Potentials, construction and operation accounted for around 50% of the overall impact in all scenarios. In these categories, the appreciable contribution of operation to the overall impact was mainly due to the use of fossil fuels for electricity production and to gases emissions (i.e. NO_x and SO_2) generated by power plants (Turconi et al., 2013). As far as Global Warming and Photochemical Oxidation Potentials are concerned, direct GHG emissions, construction and operation phases contributed equally to the overall impact in scenarios S2 and S3 (CW system coupled with gravel and graphite-based anode MFCs, respectively). On the contrary, in scenario 1 (conventional CW system) the contribution of direct GHG emissions was around 45% of the total environmental impact for the above-mentioned impact categories. It was attributed to MFCs capability of reducing methane released to the atmosphere during wastewater treatment under anaerobic conditions. In fact, in these systems bacteria involved in bioelectrochemical processes use organic matter (e.g. acetate) as a substrate, reducing the availability of the carbon source for methanogenic bacteria (Rizzo et al., 2013). For all scenarios, the contribution of operation phase to the overall impact only predominated in Ozone Layer Depletion impact category (around 60% of the total impact). Moreover, electricity produced by MFCs had a negligible impact in all considered impact categories. In all scenarios, using electricity produced by MFCs instead of electricity supplied by the grid would reduce potential environmental impact by around 3% in all impact categories.

Finally, CW system coupled with graphite-based anode MFC appeared as the best alternative to reduce CW surface requirements (by around 20%, Table 7.3) from an environmental perspective.
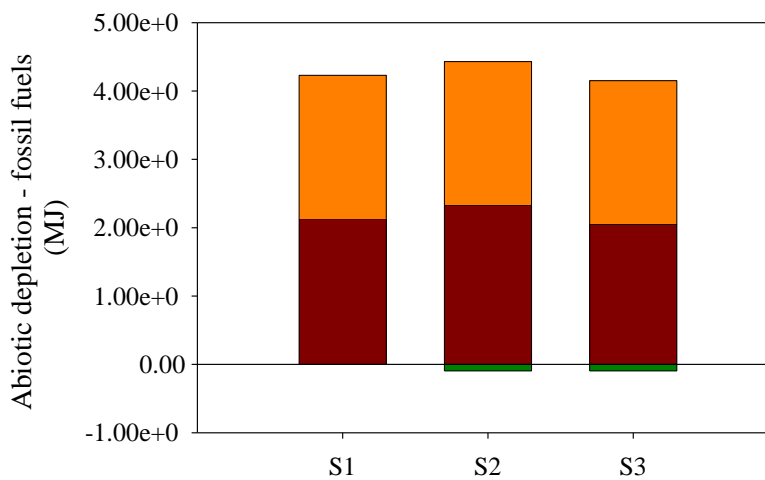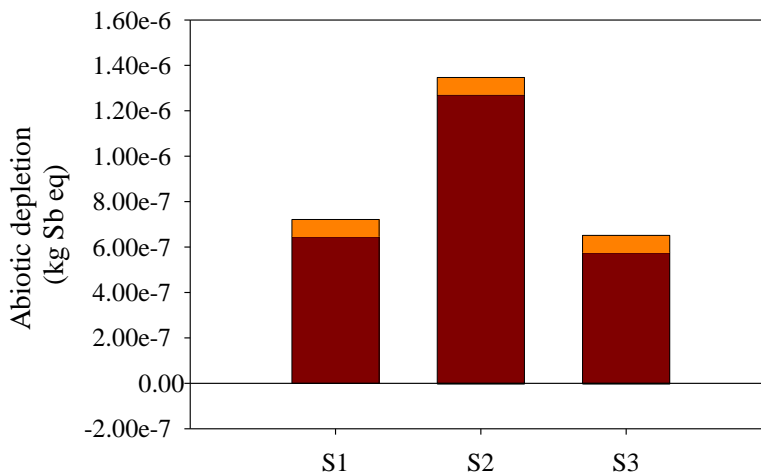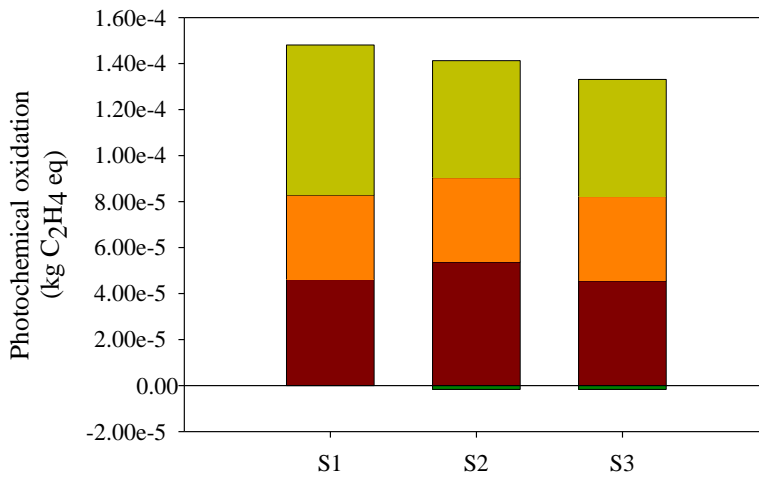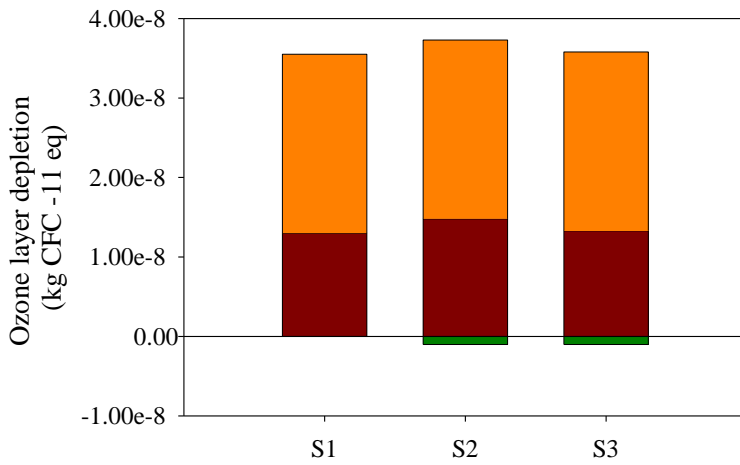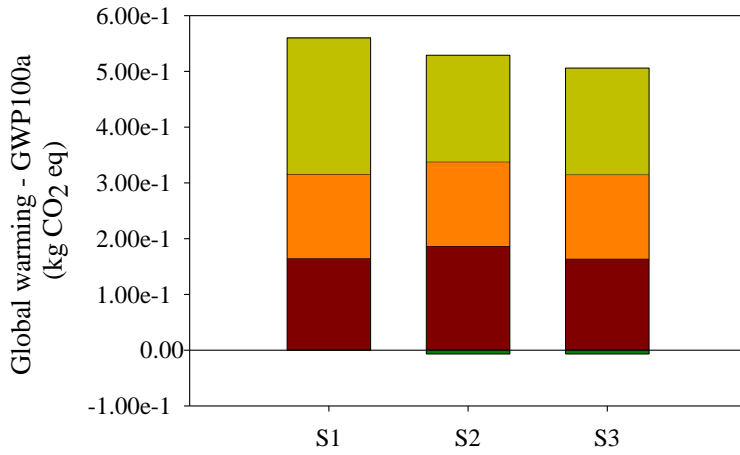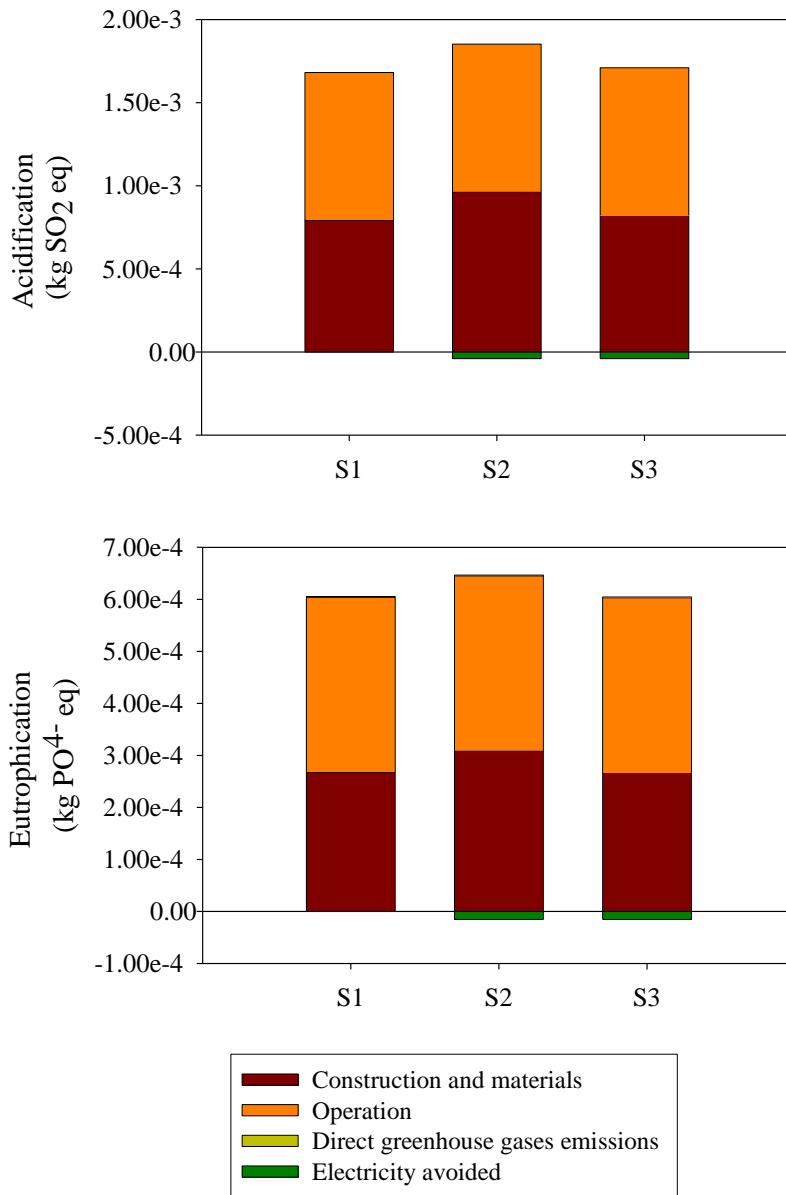
Figure 7.2 Potential environmental impacts for the three scenarios. Values are referred to the functional unit (1 m³ of water). S1: conventional CW system (without MFC); S2: CW system coupled with a gravel-based anode MFC; S3: CW system coupled with a graphite-based anode MFC

7.3.2 Sensitivity analysis

The results of the sensitivity analysis are shown in Figure 7.3. As mentioned above, it took into account two parameters: i) the k_A in scenarios S2 and S3 (CW systems with gravel and graphite-based anode MFCs, respectively); and ii) the electricity produced by the MFCs in scenarios S2 and S3 (CW systems with gravel and graphite-based anode MFCs, respectively).

Regarding the k_A, the results showed how increasing this parameter (to 0.138 and 0.162 m d^{-1}) would slightly reduce the environmental impact (by up to 10%, as compared to the base cases – 0.092 and 0.098 m d^{-1}) in all impact categories with the exception of Abiotic Depletion Potential. For this impact category, the reduction in scenario S2 accounted for around 25% as compared to the base cases (0.092 m d^{-1}). Nevertheless, scenario S2 remained the most abiotic depleting alternative.

Concerning the electricity produced by MFCs, the sensitivity analysis showed that increasing the electricity produced (to 40 Wh m^{-3} and 70 Wh m^3) would reduce all environmental indicators by 1-10% as compared to the base cases (14.4 Wh m^{-3}).

Consequently, it can be concluded that the results of the LCA are robust and not strongly dependent on the assumptions considered in this study.
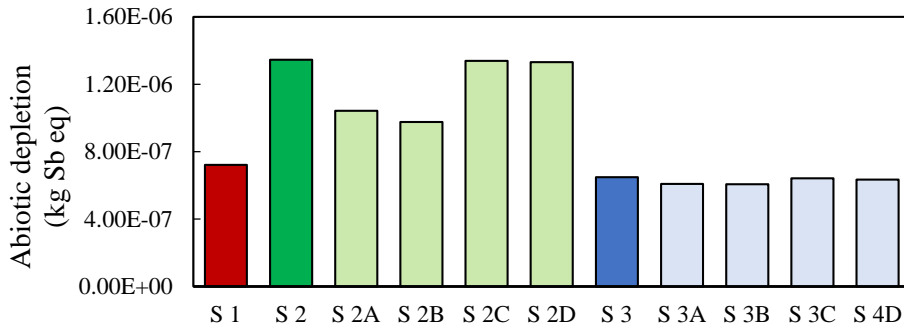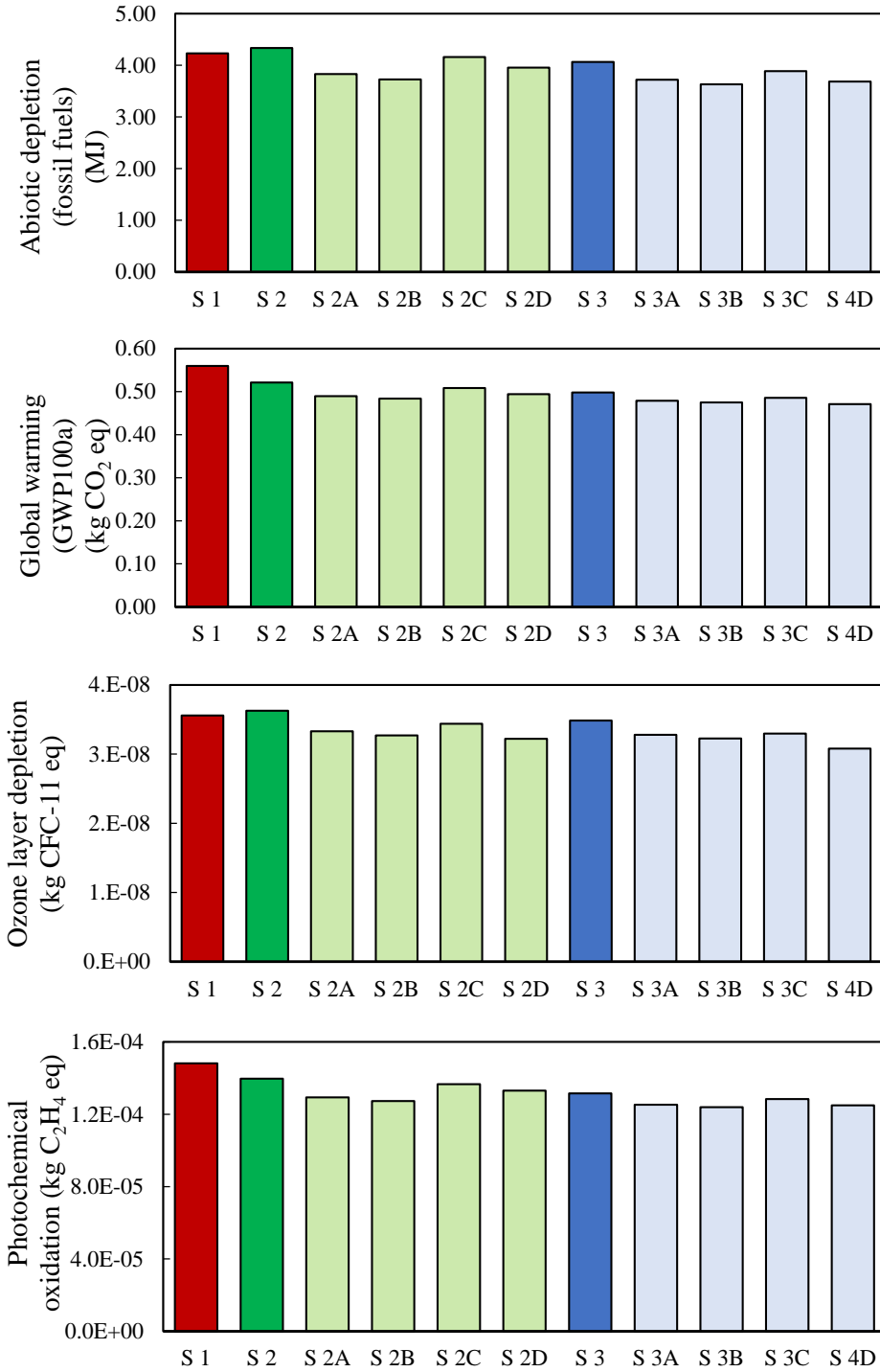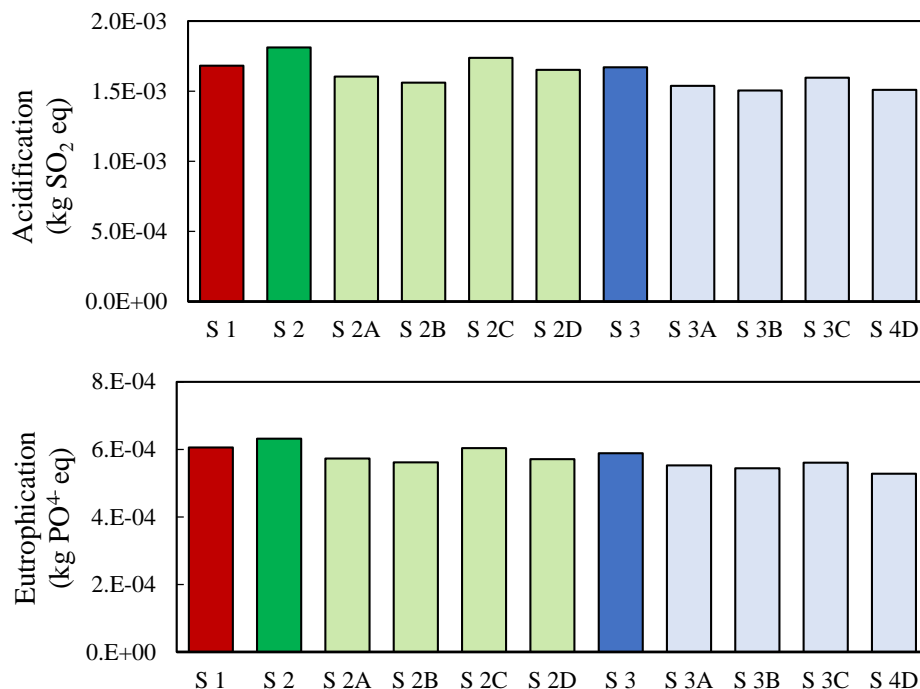
Figure 7.3 Results of the sensitivity analysis on the potential environmental impacts for the considered scenarios (Scenarios are defined in Table 7.3). Values are referred to the functional unit (1 m³ of water)

7.3.3 Economic assessment

The results of the economic assessment are summarised in Table 7.4. The capital cost of conventional CW system (scenario S1) was around 430 € p.e.$^{-1}$, which is in agreement with previous studies (Masi and Bresciani, 2013; Puigagut et al., 2007b). The CW system coupled with a gravel-based anode MFC (scenario S2) appeared as the most expensive alternative, followed by the CW system coupled with a graphite-based anode MFC (scenario S3). In particular, CW systems coupled with MFCs (scenario S2 and S3) showed to be from 1.4 to 1.6 times more expensive than the conventional CW system. It was mainly due to the high cost of materials (i.e. graphite and steel) used for microbial fuel cells implementation. In the case of scenarios with lower specific area requirement considered in the sensitivity analysis (scenarios S2A, S2B, S3A and S3B, Table 7.3), the capital costs were similar to the conventional CW system's cost (scenario S1). Thus, CW systems

coupled with high performance MFCs would be competitive with conventional CWs in terms of costs.

Table 7.4 Capital costs of the considered scenarios expressed in terms of euros per population equivalent

Scenarios [a]	Microbial fuel cells		Capital cost
	Anode	Cathode	$€\,p.e.^{-1}$
S1	-	-	432
S2 (base case)	Gravel	Graphite	726
S2A	Gravel	Graphite	518
S2B	Gravel	Graphite	488
S3 (base case)	Graphite	Graphite	639
S3A	Graphite	Graphite	470
S3B	Graphite	Graphite	445

[a] Scenarios are defined in Table 7.3

7.4 Conclusions

The CW systems coupled with MFCs are an appropriate solution for wastewater treatment in small communities which may help to reduce surface requirements, while keeping the environmental impacts low.

The CW system coupled with a graphite-based anode MFC appeared as the most environmentally friendly solution which could replace conventional CWs reducing system footprint by up to 20%.

The CW system coupled with a graphite-based anode MFC showed to be around 1.5 times more expensive than the conventional CW system. The cost of MFC-based CW would be competitive with conventional CW only under higher treatment performances of MFC than those currently attained.

For the purpose of reducing costs, cheaper materials should be investigated for MFCs implementation in CW systems.

Regarding the future research needs, an environmental and economic analysis of a full-scale CWs system coupled with MFCs should be carried out using data obtained during a long-term monitoring (e.g. MFCs lifespan,

electricity generated by MFCs, wastewater treatment efficiency, GHG emissions, costs). Moreover, a comparison with other intensified CW systems (e.g. aerated CWs and MFCs implemented in saturated vertical flow CWs) should be also addressed.

Discussion

This thesis aimed at studying and optimizing the synergy between constructed wetlands and microbial fuel cells. In this chapter results obtained from all the experiments conducted are gathered and discussed in order to become a summary of the knowledge in CW-MFCs that was generated during the thesis. Furthermore, the resulting optimal architecture and operational conditions are presented in order to establish some design criteria that maximize the benefits obtained from CW-MFCs.

The discussion chapter follows the same structure as the whole thesis. Initially, the optimal architecture and operational conditions of both constructed wetlands and microbial fuel cells are discussed. Then, the demonstrated environmental applications of CW-MFCs are presented:

electricity generation, improvement of wastewater treatment rates and organic matter and clogging assessment. Finally, LCA results and costs study provide an overview of CW-MFCs environmental and economic impacts.

8.1 CWs' operational conditions and MFCs' architecture

CW-MFCs constitute a synergy between two different technologies: constructed wetlands and microbial fuel cells. Therefore, both architecture and operation of these technologies must be reconsidered in order to obtain the maximum benefits from their synergy.

8.1.1 Constructed wetlands operational conditions

In this section the influence of HSSF CWs operational conditions such as hydraulic regime, primary treatment and the presence of plants in CW-MFCs performance are discussed.

Constructed wetlands (CW) are natural wastewater treatment systems in which wastewater is treated by means of physical, chemical and biological processes taking place within the treatment bed (García et al., 2010). Regarding wastewater flow characteristics, there are three main types of treatment wetlands (Kadlec and Wallace, 2009): Free water surface (FWS) wetlands, Vertical subsurface flow (VF) wetlands and Horizontal subsurface flow (HSSF) wetlands.

HSSF constructed wetlands, have some attractive advantages such as low cost operation and maintenance, low energy requirements and good landscape integration (García et al., 2003). In HSSF CWs, water flows horizontally and below the surface of the granular medium. Although the system is mainly anaerobic, the very upper part of the wetland remains under aerobic conditions because its close contact with the atmosphere giving maximum redox gradients of 0.41 V vs SHE between the upper and the deeper layers (Chapter 4.1). As a result, natural redox gradients in HSSF CWs could be exploited to produce energy via MFC implementation. With CW-MFCs, energy can be harvested directly from wastewater in the form of electricity (Chapter 4). In order to ensure the use of the anode as the final electron acceptor by electrochemical active microorganisms, no acceptor with

higher redox potential shall be present in their vicinity. In contrast with VF constructed wetlands, HSSF CWs are permanently flooded and therefore there is no oxygen competing for the electrons within the anodic volume which maximizes the organic matter to electricity conversion rate. Therefore, HSSF CWs constitute an optimal environment for MFCs implementation.

However, due to their predominant anaerobic nature, HSSF CWs have relatively large surface requirements when compared to intensive technologies (such as activated sludge-based treatment systems), which is one of its major drawbacks. Over the past years, research in HSSF CWs has focused on the improvement of treatment performances and the reduction of surface requirements. Among the strategies to increase CWs performances, forced (or active) aeration has been recently suggested as an efficient way to improve removal of organic matter and reduced nitrogen species (Austin and Nivala, 2009; Wu et al., 2014). Since the 1990s, active aerated systems have shown interesting results, leading to an increase of removal rates compared to passive systems (Nivala et al., 2013) resulting in the reduction of the required treatment surface. However, active aeration results in a significant increase in energy consumption during operation when compared to traditional HSSF CWs designs. As will be further discussed, the implementation of MFCs in CWs constitutes an alternative intensive strategy that generates electricity at the same time wastewater treatment rates are improved (Chapter 7).

> HSSF CWs constitute an optimal environment for MFCs implementation

8.1.1.1   Hydraulic regime

With the aim at describing the best scenario for MFC implementation in HSSF CWs, studies addressing the influence of hydraulic regime in SSF CW were conducted. It was addressed by contrasting the results obtained from the redox profiles of the planted wetlands fed with primary settled wastewater and operated under continuous and discontinuous hydraulic regime (redox

potentials were measured at 5, 15 and 25 cm below the design water level) (Chapter 4.1).

Our results suggest that the redox conditions at the surface of the wetland (up to 5 cm depth) were unaffected by the sort of hydraulic regime applied, presenting redox values of ca. +200 mV to SHE. However, the wetland operated under discontinuous flow regime presented higher redox conditions either at the middle and bottom sections of the wetland (15 and 25 cm depth, respectively) when compared to the system operated under continuous flow. More precisely, redox conditions at the bottom of the wetlands were about 125 mV higher for discontinuous flow when compared to continuous flow (Chapter 4.1). Furthermore, discontinuous flow wetlands not only showed higher daily average redox conditions at middle depths when compared to continuous flow wetlands but also showed higher daily variation of redox. This was a consequence of the discontinuous feeding (3 times per day). As a consequence of the lower redox conditions at the bottom layers of the wetland, continuous planted systems were subjected to higher redox gradient between the deeper and the upper layers. Therefore, based on redox measurements, continuous regime constitutes the best hydraulic regime for MFC implementation.

Continuous flow regime constitutes the best hydraulic regime for MFC implementation

8.1.1.2   Primary treatment

One of the main problems of constructed wetlands is clogging (Pedescoll et al. 2011a). To prevent it, primary treatments are applied to wastewater. Generally, physical treatments such as settlers or Imhoff tanks are used. However, recently other technologies such as hydrolytic upflow sludge blanket (HUSB) reactors are being considered (Pedescoll et al. 2011b). A HUSB reactor prevents methane formation during organic matter hydrolysis due to a low HRT when compared to conventional anaerobic digesters (Ligero et al., 2001b). Moreover, HUSB reactors have the advantage over

conventional settling of providing a higher concentration of biodegradable substrates (such as acetate) (Gonçalves et al., 1994) which can be easily removed in HSSF CWs. Therefore, HUSB reactors as a primary treatment are of special interest in the context of MFC implemented in HSSF CW. Accordingly, HUSB reactors will provide a higher concentration of rapidly biodegradable substrate when compared to conventional settling, thus providing higher amount of fuel for MFC. To determine the optimal primary treatment for MFCs implementation, redox potential and voltage were measured from 4 MFCs implemented in two different wetlands: the first one was fed with primary settled wastewater (settler line) while the second one received wastewater from the HUSB reactor (HUSB line) (Chapter 4.2). Redox potentials in both wetlands followed a very similar and conservative pattern. Redox potential at the anode (15 cm below the design water level) was very constant and about -220 mV for the HUSB and settler lines, respectively, without significant differences among treatment lines. Overall, redox gradients between the anode and the cathode ranged between 400 and 500 mV, regardless the experimental line considered and, thus, no significant differences were recorded among experimental conditions neither in terms of redox potential nor in terms of current production.

Even though no statistical differences were found between lines, MFCs implemented in CWs receiving wastewater from an HUSB reactor showed a better performance along both periods (maximum cell voltages and both current and power densities). To this regard, the anaerobic nature of this primary treatment may have enhanced the presence of anaerobic microorganisms, such as *Geobacter*, in the system which would have led to better electrical performances (Chapter 4.2). Moreover, the presence of significantly higher concentrations of organic matter in the vicinity of the cells may have also contributed to the higher voltages obtained from the HUSB_MFC. It is also important to point out that authors believe that one of the reasons behind the lack of significant differences among treatment lines concerning the average cell voltage recorded was the high oxygen limitation at the cathode (Chapter 4.2).

The influence of the primary treatment was also addressed in terms of the microbial community established on gravel (placed around the anode), the anode (graphite material from open and closed circuit MFC) and from primary

treated inlet wastewater from both the settler and HUSB reactor lines. Methanogenic population ranged from $0.6 \cdot 10^6$ *mcrA* gene copies g^{-1} to $1.2 \cdot 10^6$ *mcrA* gene copies g^{-1} (accounting for 0.22 to 0.49 % of total microbial populations) in graphite samples from active circuits of the settler and HUSB line, respectively. Significantly higher diversity was encountered for the HUSB line samples when compared to the settler line based on certain diversity estimators such as OTU numbers (640-775), Chao-1 (837-987), Shannon-Wienner (5.0-5.5), and even a higher evenness index (0.23-0.38) (Chapter 4.4).

Regarding the delta-proteobacteria class a remarkable feature is the relative predominance of the Geobacteraceae family in gravel and graphite samples of the HUSB line (19% and 5% of relative abundance for the graphite of active MFC and gravel samples, respectively). Within the Geobacteraceae family it is of special interest the high relative abundance of one OTU belonging to *Geobacter* in active MFC of the HUSB line (from 13 to 16%). In the case of gravel and graphite samples from the settler line, Geobacteraceae were not only less favoured (below 2%), but even the detected Geobacteraceae OTUs were different from that of the OTUs found in samples from the HUSB line. Promoting hydrolytic conditions in the HUSB line, the establishment of anaerobic conditions was favoured in wastewater inflow. Therefore, HUSB pre-treatment could favour *Geobacter* enrichment and activity outcompeting certain microbial populations on gravel and anode material in MFC-CW (Chapter 4.4).

In conclusion, hydrolytic primary treatments are the best option tested to be applied in a CW-MFC treatment plant in order to supply easily biodegradable organic compounds to the MFC and foster a more reduced environment within the treatment bed. Under these conditions a higher predominance of bacteria from the Geobacteraceae family was found in anodic biofilms.

> HUSB reactor was the best primary treatment to be applied in CW-MFCs. When implemented, the growth of bacteria from the Geobacteraceae family was fostered in anodic biofilms

> Although being a non-conductive material, gravel constitutes a suitable surface for bacteria from the Geobacteraceae family to establish

8.1.1.3 Presence of plants

The influence of plants in CW-MFCs was studied during two different experimental periods. During the first one, the effect of plants was assessed in terms of redox profiles generated along the wetland depth (5, 15 and 25 cm below the design water table). During this period, wetlands were operated with primary settled wastewater. On the other hand, during the second experimental period, when HSSF CWs were operated with a HUSB reactor as a primary treatment, MFCs voltage generation and water table variations within the wetland were monitored. Although both investigations were conducted in the same pilot plant, both periods are not comparable due to the different primary treatments applied but especially due to different growth state of macrophytes. First experiments were conducted after 6 months of plant operation while the second experimental campaign was conducted after 3.5 years of plant operation. As a consequence, during the second campaign plants had a principal effect on systems performance which was not detected during the first one.

The first sampling campaign was conducted in September 2011 (after six months of plant operation, when the plant root system already reached the bottom of the wetland). The influence of vegetation was addressed by comparing the results obtained from the planted vs unplanted wetlands operated under continuous flow regime. In this study, redox conditions were clearly unaffected by the presence of plants at 5 cm and at 25 cm depth. However, at 15 cm depth the redox was lower (between 100 and 125 mV lower) for the planted wetlands than for the unplanted. Authors believe that, since planted wetlands have been described to enhance both bacterial density and biomass (Collins et al., 2004; Gagnon et al., 2007), the redox conditions in the bulk liquid (which is actually the place where redox was measured) could be lower as a result of a higher bacterial activity. Lower redox potentials would lead to higher redox gradients through the treatment bed and therefore, to

higher voltages produced by CW-MFCs. As it was later demonstrated the effect of the presence of macrophytes in that study was underestimated to some extent due to the immaturity of the systems.

During the second experimental campaign, the effect of macrophytes on power production was evaluated for 7 days by covering two wetlands using a synthetic fabric which allowed air exchange but prevented 98% of light penetration. Before the coverage, voltage followed a cyclical daily pattern due to intense water level variation caused by evapotranspiration. However, when plants were covered water variation decreased and voltage patterns changed from variable to constant. A reduction of -45% and -71% was detected in daily mean and maximum power densities generated, respectively. In view of these results MFCs performance was mainly affected by evapotranspiration which caused water table fluctuations. As a consequence, redox conditions at the cathode and therefore, voltage generated by CW-MFCs followed the same cyclical daily pattern (Chapter 4.2).

Even though it was demonstrated that there was an influence of macrophytes on the electrical output, this influence was, actually a consequence of water table variations which, in turn, were a consequence of evapotranspiration. However, it is described that plants also supply oxygen and carbon based compounds through the roots. Therefore, the oxygen may have an influence on the redox potential within the wetland, affecting MFCs performance by increasing conditions at the cathode (positive effect) or at the anode (negative effect). At the same time, the easily biodegradable compounds given to the system (root exhudates), could be used by microbial communities in the anode to produce electricity from their oxidation (positive effect). By means of the experimental set-up used in this experiment, the influence of the oxygen and exhudates supplied by roots, could not be detected. It could be suggested that, the very reduced environment fostered by the anaerobic pretreatment and also the high concentrations of organic matter inside the wetland, may have masked the effects of macrophytes and therefore, an appropriate design should be implemented to test this potential influence that plants may have on MFCs performance.

As it is further discussed (section 8.1.2) the architecture of MFCs must cope with the effect of evapotranspiration caused by plants thus preventing the variation of oxidized conditions at the cathode along the day.

> The architecture of MFCs must cope with the effect of evapotranspiration caused by plants thus preventing the variation of oxidized conditions at the cathode along the day

8.1.2   MFCs' architecture

In this section the influence of MFCs architecture such as the relative distance between cathode and water table, the distance between electrodes, the surface cathode to anode ratio, the anodic material and the external resistance are discussed.

8.1.2.1   Relative distance between cathode and water table

As it has been pointed out, evapotranspiration caused by plants induces marked daily variations on water level within the treatment bed (Chapter 4.3) and therefore, has a notable influence on wetland's redox conditions and cell voltage in CW-MFCs. In order to determine the best relative cathode position in relation to the water table, voltages were monitored during two consecutive periods: when cathode was located (1) 5 cm below the design water level and (2) at the same design water level. This experiment was conducted after 2 years of plant operation (plants were very mature) and used a HUSB reactor as a primary treatment. The effect of evapotranspiration was clearly seen along both periods. Although the water level inside the wetlands was set to be 30 cm, significant water level variations from the design value were observed all along the study period. Consequently, when the cathode was located at 5 cm depth, water table fluctuations left it exposed to the atmosphere during the central hours of the day. When the cathode was air-exposed, oxygen availability increased and favored the current generation due to an increase of the cell voltage. Contrarily, during night hours, when the water table increased to the design level, the cathode was

submerged thus there was no redox gradient between electrodes and no current was produced. Accordingly, voltages were generally recorded from noon to about 6 pm. During the second experimental period (cathode placed at the design water level) the pattern was inverted and voltages were recorded during hours were evapotranspiration was not intense. Accordingly, cell voltages were generated during most part of the night and dropped during daylight when the cathode was well above the water level and reached a drying state that hampered any electrons flow (short-circuit conditions). Our results suggest that the main factor governing the cell voltage in our system was the availability of oxygen at the cathode which was related to water level variation caused by plants evapotranspiration.

The main configuration for MFC implementation in HSSF CWs relies on placing a cathode at the surface of the system while the anode remains buried in the deeper zone of the treatment bed. Notable fluctuations of the water table caused by plants evapotranspiration may vary MFC performance on daily and seasonal terms by changing the availability of oxygen at the cathode. From our results we can conclude that only when the cathode was slightly above water level (position around 1 to 2 cm above the water level) the cell voltage was maximized. These results suggested that, even though it was reported that the upper water layer of HSSF CWs is under higher redox conditions (Chapter 4.1), the real oxidized layer from which we can benefit by implementing MFC is of very little extend. Consequently, when the cathode was even slightly submerged there was not enough oxygen in the cathodic zone for MFC to produce any significant current. Performance of cathodes is therefore, considered as the main source for MFC performance limitation. An analysis based on impedance was also performed (polarization curves under optimal and suboptimal cathode positions) and showed that cathode position under optimal conditions reduces the internal resistance of the system and, therefore, maximizes cell performance.

For all that, in order to avoid cathode limitation in MFC implemented in CW, cell architecture shall address the possibility to cope with intense water level variations. To this regard, a cathode based on a thick layer of graphite placed at the upper part of the treatment bed will allow the CW-MFC to have always an active cathode zone for oxygen reduction, regardless the water level within the wetland. According to our results, water level can vary up to 10 cm

from the design water level (Chapter 4.2). Therefore, the thickness of the graphite layer shall be, at least, 10 cm.

> Only when the cathode was around 1 to 2 cm above water level the cell voltage was maximized

8.1.2.2 Distance between electrodes

Distance between electrodes is also one of the factors adding internal resistance to the system and, as a consequence, the higher the distance between electrodes the lower the power density recorded in conventional MFCs (Ghangrekar and Shinde, 2007). However, to optimize MFCs performance the redox gradient existing between the anode and the cathode must be maximized. Redox potential within the CW treatment bed is subjected to great spatial and temporal variation, which is caused, in turn, by several factors including the presence of plants, fluctuations in the water level due to evapotranspiration, light intensity and temperature (Wießner et al., 2005; Dušek et al., 2008; Białowiec et al., 2012; García et al., 2010). In general terms, redox potential variation in SSF CWs is accepted to be of greater intensity in depth rather than across the length of the bed (Garcia et al., 2003). As a consequence, the placement of electrodes within the treatment bed must consider the maximization of the redox gradient between electrodes and the minimization of the internal resistances generated due to the distance between them.

The effect of the distance between electrodes on the electricity production was addressed in a lab-scale CW-MFC (Chapter 4.3). The lab-scale CW-MFC's anode chamber was designed to simulate a core of a shallow wetland gravel bed in which the electrons derived from exoelectrogens organic matter oxidation would be transferred to the electron collector (stainless steel mesh). Voltage generated by the gravel-based lab-scale CW-MFC decreased with the distance between electrodes and a linear relationship could be established between both parameters. Maximum voltages were obtained at the minimal distance tested (10 cm).

The analysis of redox gradients along the depth of SSF CWs (5, 15 and 25 cm depth) was used to describe the optimal scenario to maximize the energy production of CW-MFCs (Chapter 4.1). The daily average redox gradient along the depth of the wetland was maximal between the surface (5 cm depth) of the wetland and the bottom (25 cm depth) and, to a lesser extent, between the surface and the middle depth (15 cm depth), regardless the operational or design conditions applied. However, the redox gradient between the middle depth and the bottom was very small regardless the experimental conditions tested. Therefore, although the maximum redox gradient was obtained between 5 and 25 cm depth, the difference between 25 and 15 so small that it can be concluded that all the bed depth is almost equally reduced.

Overall, as it has been discussed, the cathode must cope with the effect of evapotranspiration by guaranteeing the constant contact of the water table with the electrode (Chapter 4.3). Also, the distance between electrodes must be minimized in order to reduce the internal resistance of the system (Chapter 4.3). Therefore, given that all the bed is equally reduced, the anode can be placed the closest to the cathode but, also considering water level fluctuations, it must be kept deep enough to avoid its contact with the atmosphere due to the evapotranspiration effect (from ca. 10 cm).

> The anode must be placed as close as possible to the cathode but deep enough to avoid its contact with the atmosphere due to the evapotranspiration effect (from ca. 10 cm)

8.1.2.3 Surface cathode to anode ratio

Voltage generated by MFCs is always diminished by overpotentials and ohmic losses which can occur both in the anodic and the cathodic compartments (Logan et al., 2006). Cathodic overpotentials are main contributors to overall performance losses in MFCs (Rismani-Yazdi et al., 2008) due to the slow kinetics of the oxygen reduction at graphite electrodes (Gil et al., 2003). Therefore, the cathode can constitute one of the most

limiting steps of the biolectrochemichal process (Chapter 4.3) thus hindering the effect of other parameters affecting cell performance, such as the primary treatment applied or the anodic material used (Chapter 4.2 and 5). To study CW-MFCs performance, it is crucial to ensure that the cathode is not limiting the system. One of the strategies to lower cathodic overpotentials is increasing the electrode surface area (Logan et al., 2006). Accordingly, experiments to determine the optimal cathode to anode surface ratio (C:A) were conducted both to the pilot plant and to the lab-scale MFCs. Results show that voltage increased together with the cathodic area until the C/A ratio was 4 and 3.2 cm^2/cm^2 for the pilot plant (graphite-based cathode and graphite-based anode) and the lab-scale CW-MFCs (graphite-based cathode and gravel-based anode), respectively. From then on, although in both cases the ratio was further increased (up to 5 and 4 cm^2/cm^2, respectively) either voltage generated decreased (pilot plant CW-MFC) or no statistical differences were found between voltages generated at the two last steps (lab-scale CW-MFC). Hence, results obtained in this experiment suggested that, when wastewater was used as substrate, the ratios between 3 and 4 cm^2/cm^2 were enough to avoid cathodic limitation.

However, the optimal anode to cathode surface ratio is set-up dependent. Accordingly, different configurations and materials would lead to diverse overpotentials that must be specifically quantified. Given the importance of the cathode limitation regarding CW-MFCs performance when anodic reactions are assessed, previous investigations must be conducted to each CW-MFC type in order to ensure that cathodic reactions are not the limiting factor. If similar configurations and same materials than those presented in this thesis are used, the anode to cathode surface ratios stablished can become reference values.

> Cathode to anode ratio of ca. 4 cm^2/cm^2 was enough to avoid cathodic limitation in CW-MFCs

8.1.2.4   Anodic materials

The electrode material used in MFC architecture is a key parameter regarding their performance. Good electrical conductivity is one of the most important properties to optimize MFCs functioning (Zhou et al., 2011). Therefore, non-conductive materials are rarely used as anodic materials in conventional MFCs due to their incapacity to collect and transfer electrons. However, the presence of exoelectrogens in gravel biofilms of CW-MFCs demonstrates the capacity of electroactive biofilms to establish on non-conductive materials (Chapter 4.2). Accordingly, in order to test the influence of the anode material we compared the effect of using a very common conductive electrode material (graphite) with a non-conductive material typically used as filling media in constructed wetlands (gravel) (Chapter 5). Both materials were wrapped with stainless steel mesh that worked as the electron collector.

The effect of the anodic material was compared both in terms of removal efficiencies of the principal wastewater pollutants and also in terms of the electricity generated in the investigated lab-scale CW-MFCs. The electricity generated in gravel and graphite based CW-MFCs was recorded during two different experimental periods. The first experimental period was conducted just after the construction of the lab-scale CW-MFCs, when systems were completely clean (a start-up period of ca. 10 days was left). During this period, although there was no difference between materials in terms of the voltage patterns followed and between removal efficiencies detected, due to the higher conductivity of the anodic material used, graphite MFCs generated higher voltages and intensities. More precisely, after 20h of hydraulic retention time graphite based CW-MFCs produced intensities $163\pm92\%$ higher than gravel based systems.

The second experiment was conducted after 8 months of CW-MFCs operation when biofilms were highly developed and systems were partially clogged. In this second experiment, graphite and gravel-based anode CW-MFCs did not produce any significant difference between their removal efficiencies for any of the analyzed water quality parameters. Unexpectedly, same conclusions can be outlined regarding cell voltages recorded. Since it is widely accepted that the conductivity of the anodic material is positively

related to the cell performance (Logan, 2008), results obtained from this experiments shall be explained by means of a significant limitation in graphite-based systems. This limitation must have hindered graphite-based CW-MFC potential to the point that no differences can be seen between a conductive media such as graphite and a non-conductive media such as gravel based MFCs.

As it has been outlined, MFCs used in the second experiment were previously operated during 8 months before it was conducted. Domestic wastewater is known to have high content of suspended solids (Pedescoll et al., 2011b) which progressively accumulates within the system. This accumulation of solids decreased CW-MFCs performance in gravel based systems (Chapter 6.2) thus it might also have limited the capacity of the graphite systems to transfer electrons to the external circuit.

> Solids accumulation within the anode might have caused a significant limitation in graphite-based systems to the point that no differences were seen between a graphite and gravel based CW-MFC performance

8.1.2.5   External resistance

The external resistance plays also a role regarding MFC performance (Aelterman et al., 2008). Different configurations (materials, dimensions, substrates, etc..) are linked to different internal resistances and hence, different optimal external resistances (Logan, 2008). Overall, it is generally accepted that the lower the external resistance the higher the current generated and the higher the organic matter removal rate (Aelterman et al., 2008; Gil et al., 2003; Katuri et al., 2011). The external resistance that maximized the organic matter difference between the effluent of the connected (MFC[+]) and non-connected (MFC[-]) systems was studied by means of gravel-based lab-scale MFCs. Five different external resistances were tested: 50, 220, 402, 604 and 1000 ohms. The highest significant differences between connected and not-connected systems were recorded at 220 ohms of external resistance both for total or

soluble COD. Furthermore, the difference between MFC$^+$ and MFC$^-$ outlet concentrations tended to decrease with the external load applied (Chapter 5). Therefore, 220 ohms was proven to be the optimal external resistance for CW-MFCs treatment efficiency improvement.

> 220 ohms was the optimal external resistance for CW-MFCs treatment efficiency improvement

8.1.3   Microbial-derived limitations

CW-MFCs limitations derived from the operation of CWs and from the architecture of MFCs have been already discussed (sections 8.1.1 and 8.1.2). In this section microbial-derived limitations influencing CW-MFCs performance are also discussed.

HSSF CWs constitute a complex environment in which microorganisms growth is highly influenced by external factors such as temperature, pH, salinity, plants, availability of organic carbon or even the presence of inhibiting substances (Faulwetter et al., 2009). Such complexity and variability which is described to affect nitrogen and sulphate reducing bacteria could also constitute a constraint to exoelectrogenic bacteria, hindering their optimal growth and their ability to transform organic substrates into electricity. Furthermore, this complexity enables the occurrence of very different degradation pathways that make HSSF CW a very competitive environment for electrigens to establish (Chapter 4.2). There is also a presence of a large number of different electron acceptors that become an alternative to the electrode thus decreasing the quantity of electrons transformed to electricity. Moreover, wastewater has a significant content of organic matter potentially used as a carbon/electron source although it is composed by complex substrates. It is known that electrigens are only able to oxidize simple carbohydrates (Kiely et al., 2011) and therefore, the previous hydrolysis of long-chain organics, which relies on fermentative bacteria, can be a limiting step for these bioelectrochemical systems (Chapter 4.4). Accordingly MFCs operated with acetate (a simple carbohydrate) showed

significantly higher power densities (W/m^3) and energy recoveries (KWh/kg COD) than those operated with wastewater (Ge et al., 2014) indicating the clear limitation that the type of substrate represents. This limitation could be partially alleviated by the presence of electrigens able to oxidize a wide range of electron donors (Kiely et al., 2011), which was found not to be true for the electrigen studied (*Geobacter lovleyi*) (Chapter 4.4). This electrigen was predominantly colonizing anodic biofilms in CW-MFCs operated with real wastewater. Other limitations such as mass-transfer limitations due to the accumulation of non-biodegradable substrates in the gravel matrix (clogging) that hinders the electron transfer to the electrode and decreases the available surface for bacteria to utilize may also constitute a source of resistance (Chapter 6.2). But not all the limitations are focused on the anodic area, actually, as already discussed above, the reduction of O_2 at the cathode has been described to be a major constrain in CW-MFCs due to the slow kinetics of the reaction and the low availability of oxygen under suboptimal cathode position, which converts the cathode performance into the most limiting step (Chapter 4.3).

In conclusion, most of the limitations have a direct or indirect effect on the microbial community, and specifically on electrigens which are a key factor for an optimal performance of CW-MFCs. CW-MFC show limited performance when compared to more traditional MFC configurations. Accordingly, pure culture studies using conventional air-cathode MFCs fed with acetate achieved coulombic efficiencies (CE) higher than 90% (Clauwaert et al., 2007), whereas CE for CW-MFCs ranges from 10±6% to 42±14% for total COD in lab-scale graphite-based CW-MFCs (Chapter 5 and 6.1). Reported CE values for MFCs implemented in wetlands are actually lower than those here reported (between 0.05% and 3.9%) (Doherty et al., 2015). The significant limitations to which CW-MFCs are subjected were also described by means of the cell efficiency (voltage measured (E_{cell}) compared to the attainable maximum (E_{emf})) which was, in average, 13 $\pm$ 12% and 7 $\pm$ 5% using the HUSB and the settler line, respectively (Chapter 4.1). CW-MFC is a very recent technology and therefore its limitations have not been deeply characterized. Nevertheless, some of the well-defined limitations of conventional MFCs, such as those derived from bacterial and metabolic losses, could also apply to CW-MFCs. Accordingly, *G. lovleyi* was studied

under laboratory conditions to identify whether it should be considered a source for reduced performance in CW-MFC.

MFCs which were unconditioned and fed with real wastewater showed a spontaneous enrichment of exoelectrogenic bacteria promoted by the presence of a new electron acceptor within the media (the anode) (Chapter 4.2). An OUT closely similar in sequence to *Geobacter lovleyi* was found to be the predominant species in anodic CW-MFC biofilms (Chapter 4.2). In our work, we have found that *G. lovleyi* was not able to degrade a wide range of electron acceptors, which probably might be a current limitation given the wide range of organic intermediates derived from anaerobic routes taking place within constructed wetlands (Kadlec and Wallace, 2009). The enrichment of *Geobacter* species in wastewater and fermentation products fed systems relies on their capacity to attach to electrodes and generate electroactive biofilms. However, to succeed in the process, bacteria need both energy and carbon sources to support their growth. Therefore, the lack of electron donors or acceptor versatility could represent an important source of limitation for CW-MFCs. Accordingly*,* neither lactate nor formate supported *G. lovleyi* growth (Chapter 4.4). Contrarily, acetate was demonstrated to be a suitable electron and carbon source for *G. lovleyi* either with fumarate or Fe(III) citrate as electron acceptors. Acetate is the key intermediate in anaerobic digestion and therefore it is always present in HSSF CW. Therefore, the acetate rich environment in HSSF CW guarantees *G. lovleyi* survival. However, in our study we have found that *G.lovleyi* requires the presence of a strong reductant agent (such as cysteine) and the complete absence of oxygen in order to grow. This intolerance to oxygen could become a major limitation for CW-MFCs since HSSF CW are planted systems and oxygen is released from the macrophytes root system (Tanner, 2001). Moreover, plants evapotranspiration causes a daily fluctuation of the water level which stimulates the presence of oxygen within the gravel media leading to higher redox potentials in some areas of the treatment bed (Chapter 4.3). Therefore, the anodic colonization by oxygen intolerant electrigens *(*such as *G. lovleyi)* may result in a reduction of the current generated. Finally, *G. lovleyi* was demonstrated to be not very efficient in colonizing the graphite rod electrode. Actually, the colonization was influenced by the potential poised (Chapter 4.4). As a consequence, its establishment in an HSSF CW could be affected

by the naturally fluctuating redox gradient which depends on external conditions and cannot be maintained at a constant value along time (Chapter 4.1 and 4.3). Redox potentials in deep zones of planted HSSF CW have been described to reach ca. -200 mV, resulting in a maximum generated voltage of 140 mV (Chapter 4.2). This suggests that the anode potential in CW-MFCs is negative and, as we have observed in this study, *G. lovleyi* is not very efficient in transferring electrons to the anode when it is poised at lower potentials than 450 mV (Chapter 4.4). Overall, the authors believe that MFC implemented in constructed wetlands may experience a reduction of performance whenever the dominant electricigen is *G. lovleyi* which is confirmed by the low coulombic efficiencies obtained in MECs driven by *G. lovley* which ranged from 30 to 40%, which is less than half of those estimated for *G. sulfurreducens* (~ 80%) (Chapter 4.4). Finally, it is still unknown whether CW-MFC anode conditioning with electricigen species showing higher coulombic efficiencies, with a wider range of oxidable substrates and less affected by the presence of low oxygen concentration might result in better CW-MFC performances.

8.2   CW's treatment efficiency improvement

Constructed wetlands (CWs) constitute a robust and efficient technology that, if properly designed, is able to meet the limits established in current environmental policy (Kadlec and Wallace, 2009). However, CWs are only suitable technologies for small communities (Puigagut et al., 2007b) due to their large surface requirements when compared to conventional wastewater treatment technologies (Kadlec and Wallace, 2009). Accordingly, current research efforts in wetland technology have focused on the design and implementation of intensive strategies (such as wetlands aeration, reciprocation, specific filter media, etc..) to improve nutrient and organic matter treatment efficiency as well as reduce surface requirements. However, most of the currently available intensified wetland configurations result in higher costs of operation (mostly due to higher energy consumption rates). In this context, results here reported suggest MFCs as an alternative to currently available intensive HF CWs strategies to increase their removal rates with energy generation instead of high energy consumption. Furthermore, higher organic matter degradation rates in HF CWs operated as MFCs may result in a reduction of wetland surface requirements, thus enabling more compact designs and reducing the cost for land acquisition.

One of the aims of this thesis was to quantify the extent of treatment efficiency improvement in lab-scale membrane-less MFCs simulating a core of a shallow wetland (gravel-based anode) by comparing the treatment rates of connected and unconnected CW-MFCs (CW-MFC$^+$ and CW-MFC$^-$, respectively). Although the experimental systems used cannot be fully considered as constructed wetlands due to a lack of plants and the batch loading regime, reported results might be useful for the improvement of the design and operation of constructed wetlands working as microbial fuel cells.

The obtained results strongly suggested that the operation of constructed wetlands as MFCs would increase domestic wastewater treatment performance. More precisely, the concentration of organic matter at the effluent of CW-MFC$^-$ was always statistically higher than in CW-MFC$^+$, indicating a stimulation of the organic matter oxidation in connected systems. Effluent concentrations in connected systems were 21±2 %, 18±11% and 15±16% lower than not-connected systems, for BOD_5, COD and total OC, respectively. Furthermore, our results indicate that the equivalent organic matter removed by the exoelectrogenic pathway in connected MFC (estimated from the electrons transferred through the external circuit) is much higher (25.2 ±11.1 mg O_2) than the actual difference between the connected and the unconnected MFCs (average difference of 11.7 ±11.3 mg O_2). Two hypotheses can be suggested to explain this fact. On the one hand, there might be some inorganic electro-active species interacting with the electrode that cannot be detected by means of the COD analysis. On the other hand, the second hypothesis is based on the fact that bacteria performing the anaerobic degradation of organic matter such as methane producing bacteria might be outcompeted by exoelectrogenic bacteria. Unfortunately, our experimental design did not allow us to differentiate the relative contribution of each one of the two suggested pathways. Therefore, the main route responsible for the improvement of MFC treatment efficiency here reported remains still unknown.

Although the main purpose of constructed wetlands is the removal of organic matter from wastewater, other contaminants such as nitrogen, sulphur or phosphorus are also of importance given current figures of environmental pollution and environmental policy restrictions. Like organic matter, ammonia outlet concentration was always lower in MFC$^+$ when compared to the MFC$^-$

(P<0.1), indicating a stimulation of the ammonia removal in connected systems. Regarding sulphate, inlet wastewater used constituted a sulphate-rich media. However, although not connected systems were able to remove up to 70% of the inlet sulphate, it was less efficiently removed in connected systems (ca. 57%). According to current literature, sulfides can be partially re-oxidized to sulfur and then, again, to sulphate, using the anode as the electron acceptor, thus generating electrical current in a cyclical sulfate-regenerating process (Lovley, 2006). Our results support this hypothesis since the concentration of sulfates in the anode chamber of connected MFCs was always significantly higher than those measured in unconnected MFC. Finally, phosphorus removal in our active MFC was significantly increased when compared to unconnected MFC. Phosphorus removal might have been enhanced through biological uptake rather than chemical precipitation at the cathode. However, future work on the characterization of biological phosphorus uptake in membrane-less microbial fuel cells shall be addressed to confirm the findings here reported.

Overall, our results suggest that HSSF CWs operated as MFCs is a promising strategy to improve domestic wastewater treatment efficiency (either in terms of organic matter removal, ammonia and phosphates).

> HSSF CWs operated as MFCs is a promising strategy to improve domestic wastewater treatment efficiency

8.3   COD and clogging assessment

A microbial fuel cell (MFC) is a technology that enables a bioconversion of chemical energy contained in organic/inorganic compounds into electrical energy. Besides the use of MFCs as a novel technology for green energy production, it can also be used as a biosensor tool for organic matter concentration assessment. MFCs directly provide an electrical signal that can be correlated to organic wastewater content (Peixoto et al., 2011). The main advantages resulting from the utilization of MFCs as biosensor devices are the possibility of in-situ implementation and on-line monitoring. In the context

of natural-based wastewater treatment technologies the use of a biosensor tool for the assessment of operational conditions is of key importance for plant management optimization. Both the quantity of organic matter present in wastewater (influent and effluent) and the extend of the clogging phenomena can be bioindicated by CW-MFCs.

8.3.1 COD assessment

Biochemical and biological oxygen demand (COD and BOD, respectively) are commonly used as the analytical methodologies to determine wastewater organic matter content in treatment plants. Although its precise and rapid quantification is crucial, current methodologies are time consuming, produce chemical compounds that pose a threat to the environment and require qualified personnel (Kumlanghan et al., 2007). As a consequence they are not suitable for real-time monitoring thus preventing a rapid response to contamination events (Di Lorenzo et al., 2009; Kim et al., 2003b).

Accordingly, we studied the use of a novel membrane-less CW-MFC as a suitable wastewater COD biosensor (both gravel and graphite were tested as anodic materials) (Chapter 5). Results obtained from the study showed a positive linear relationship between current generated and inlet COD concentration (from 25 to 200 mg O_2/L) after 20h of contact time. This linearity was found both for gravel and graphite based MFCs. However, the latter showed a better biosensing capacity both in terms of the correlation coefficients but also of the bioindication ranges. With the increase of contact time, both gravel and graphite based CW-MFCs showed an improvement of the quality of the linear relationship between current and inlet organic matter. Accordingly, our biosensors showed correlations with R-square values higher than 0.8 for contact times higher than 10 hours. Also bioindication ranges grew with the contact time.

Bioindication is based on the fact that a reliable relationship can be established between the current generated and the quantity of organic matter present in the media. Hence, a deep reflection is necessary concerning the parameters that can hinder its viability. First of all, the organic matter indicator and the electric signal parameter that best fit MFC bioindication characteristics must be consciously chosen (Chapter 6.1).

Furthermore, Microbial fuel cells working with wastewater and, especially, in the context of constructed wetlands, are subjected to very variable conditions which lead to variable microbial communities in anodic biofilms. This variability of biofilms can result in a variation of the electrical response of the system and the consequent reduction of sensors reproductivity. Also, as a consequence of the retention of particulate organic matter and the biofilm generation, organic substrates are accumulated within the anodic gravel matrix. This organic matter is used by exoelectrogens thus participating in the generation of electricity which does not come from inlet wastewater. Actually, bioelectrochemical response of MFCs in terms of current production was highly affected by the previous organic loads the system had received (Chapter 6.1). Both facts lead to the need for frequent system recalibration in order to reestablish their short operational stability. Therefore, longer studies should be undertaken to determine the required calibration frequency for such biosensing tools.

Finally, due to its diverse composition there are many compounds in wastewater that can be used as electron acceptors, like nitrates or sulfates, instead of the sensor's electrode (Liu and Logan, 2004). Coulombic efficiencies (CE) obtained in our experiments were always below 30% and 15% for graphite and gravel MFCs, respectively. That indicates that most of the organic matter was actually removed by means of conventional anaerobic pathways such as nitrification or sulfate-reduction. On the contrary, electricity can also be generated through direct anodic oxidation of non-organic compounds which could also hinder the bioindication capacity of the sensors. Although results presented in this study determined that abiotic reactions were not a significant part of the electricity generation (accounted for less than 2% of the electrons transferred) (Chapter 6.1), other configurations using different media must consider their potential effect. Under these conditions, MFC based sensors like the one proposed in this article, would only give a reliable signal in terms of bioindication if the composition of wastewater (including each of its specific compounds) would be proportional to the exoelectrogenically degradable OM concentration.

Although results obtained with this work indicate that linear relationships can be established between inlet COD and current generated, as it has been discussed there are several factors that can affect the precision, the

repeatability and the operational stability of the devices. Therefore, other alternative uses should be suggested for biosensor CW-MFCs. These alternatives could consider its utilization as a qualitative assessment tool which raise an alert when particularly high organic matter concentration within a media occur.

> CW-MFCs are better suited as a qualitative, range-like semi-continuous COD assessment tool

8.3.2 Clogging assessment

HSSF CWs suffer from a progressive media obstruction, the so-called clogging process which is one of its major operational problems (Nivala et al., 2012). Loss of hydraulic conductivity, creation of preferential water-flows, ponding and reduction of both treatment efficiency and system's life-span are some of the consequences of clogging (Caselles-Osorio and García, 2006; Knowles et al., 2011; Nivala et al., 2012; Wallace and Knight, 2006). A great extent of the particulate material retained within HSSF CW is of organic nature (Knowles et al., 2011; Pedescoll et al., 2011a) and hence, microbial fuel cells (MFC) could be suitable technology for indirect clogging assessment.

Actually, our results showed that sludge retention was negatively correlated with both the electrons transferred to the circuit and the voltages generated for each batch test performed. Hence, the more sludge accumulated within the MFC the lower the amount of electrons transferred. Author's hypothesis behind the reduction of current generated under higher particles accumulation is that the bacterial activity decreases due to limited substrate availability. Accordingly, the lower the pore space available within the filter media, the lower the substrate availability for exoelectrogens and the lower the amount of electrons transferred. This hypothesis is consistent with the described general functioning of bacterial activity in horizontal subsurface flow constructed wetlands (Samsó and García, 2014). Results showed that after an equivalent clogging time of ca. 5 years the MFC was no longer able to produce any significant electric charge.

Also the electric charge was dependent on the contact time, the level of correlation between the electric charge and the amount of sludge retained (or clogging time essayed) was of similar magnitude (always showing $R^2>0.9$), regardless the contact time considered. Therefore, from our results it seems that there is no specific contact time out of those here considered that results in better MFC performance in terms of clogging assessment.

To monitor/mitigate/reverse the clogging phenomena in HSSF CWs, different strategies are currently envisaged (Nivala et al., 2012). Our result suggests that, although longer study periods under more realistic conditions shall be further performed, MFC has a great potential to be used as an indirect, continuous clogging assessment tool in constructed wetlands.

> MFC has a great potential to be used as an indirect, continuous clogging assessment tool in constructed wetlands

8.4 Life cycle and economic assessment

Life Cycle Assessment (LCA) was conducted to determine the environmental impact of Microbial fuel cells (MFCs) implemented in constructed wetlands (CWs) in a real-scale treatment plant (1500 p.e.). The implementation of MFCs in HSSF CWs may lead to important benefits. First of all, it provides an energy surplus that can partially cover the energy input necessary for wastewater treatment (Chapter 4.3). Moreover, MFCs can stimulate the degradation of organic matter present in wastewater by fostering more efficient degradation pathways carried out by exoelectrogenic bacteria (Chapter 5). As a consequence, the implementation of MFCs in HSSF CWs can improve CWs treatment efficiency and reduce their surface requirement (Chapter 7). However, materials used for conventional MFCs electrodes (e.g. carbon fiber, stainless steel) are expensive materials with poor environmental performance (Liu and Cheng, 2014; Zhou et al., 2011). Therefore, although energy inputs and surface area requirement could be reduced, both costs and environmental impacts could significantly increase when implementing MFCs in CW treatment systems. In order to evaluate the environmental impacts and

the economic costs caused by HSSF CWs coupled with MFCs made of different materials, three scenarios were compared: 1) a conventional CW system (without MFC implementation); 2) a CW system coupled with a gravel-based anode MFC, and 3) a CW system coupled with a graphite-based anode MFC.

For all scenarios, the contribution of the construction and operation stages in the Abiotic Depletion impact category accounted for 88-95% and 5-12% of the total impact, respectively. With regards to Abiotic Depletion (fossil fuels), Acidification and Eutrophication Potentials, construction and operation accounted for around 50% of the overall impact in all scenarios. In these categories, the appreciable contribution of operation to the overall impact was mainly due to the use of fossil fuels for electricity production and to gases emissions (i.e. NO_x and SO_2) generated by power plants (Turconi et al., 2013). As far as Global Warming and Photochemical Oxidation Potentials are concerned, direct GHG emissions, construction and operation phases contributed equally to the overall impact in CW system coupled with gravel and graphite-based anode MFCs. On the contrary, in the scenario with conventional CW system the contribution of direct GHG emissions was around 45% of the total environmental impact for the above-mentioned impact categories. For all scenarios, the contribution of operation phase to the overall impact only predominated in Ozone Layer Depletion impact category (around 60% of the total impact).

Moreover, electricity produced by MFCs had a negligible impact in all considered impact categories. In all scenarios, using electricity produced by MFCs instead of electricity supplied by the grid would reduce potential environmental impact by around 3% in all impact categories. Moreover, increasing the electricity produced (to 40 Wh m^{-3} and 70 Wh m^{-3}) would reduce all environmental indicators by 1-10% as compared to the base cases (14.4 Wh m^{-3}). Regarding the first order rate constant for BOD removal (k$_A$), the results showed how increasing this parameter (to 0.138 and 0.162 m d^{-1}) would slightly reduce the environmental impact (by up to 10%, as compared to the base cases – 0.092 and 0.098 m d^{-1}) in all impact categories with the exception of Abiotic Depletion Potential. For this impact category, the reduction in the gravel-based anode scenario accounted for around 25% as compared to the

base cases (0.092 m d^{-1}). Nevertheless, it still remained the most abiotic depleting alternative.

Finally, CW system coupled with graphite-based anode MFC appeared as the best alternative to reduce CW surface requirements (by around 20%) from an environmental perspective.

In terms of the economic assessment, the CW system coupled with a gravel-based anode MFC appeared as the most expensive alternative, followed by the CW system coupled with a graphite-based anode MFC. In particular, CW systems coupled with MFCs (both gravel and graphite based scenarios) showed to be from 1.4 to 1.6 times more expensive than the conventional CW system. It was mainly due to the high cost of materials (i.e. graphite and steel) used for microbial fuel cells implementation. However, in the case of scenarios with lower specific area requirement, the capital costs were similar to those of the conventional CW system. Thus, CW systems coupled with high performance MFCs would be competitive with conventional CWs in terms of costs.

> CW system coupled with graphite-based anode MFC appeared as the best alternative to reduce CW surface requirements (by around 20%) from an environmental perspective

> Although the high cost of materials (graphite and stainless-steel) used for MFCs implementation, CW systems coupled with high performance MFCs would be competitive with conventional CWs in terms of costs

8.5   Energy gain

Constructed wetlands (CWs) is a technology that consumes little energy for the treatment of domestic wastewater (<0.1 KWh/m^3) (Kadlec and

Wallace, 2009). As it has been previously discussed, due to its large surface requirements (about 5 m²/PE) CWs implementation is generally restricted to the sanitation of small human settlements (<10,000 inhabitants). In order to not only overcome the large surface requirements of CWs but also to increase its treatment efficiency, active aeration has been considered a suitable strategy (Kadlec and Wallace, 2009; Austin and Nivala, 2009). However, CWs aeration increases the energy devoted to the treatment of wastewater when compared to passive, more traditional wetlands configuration. Current figures of energy consumption for completely aerated wetlands range from 0.16 to 0.49 kWh/m3 (Kadlec and Wallace, 2009; Austin and Nivala, 2009).

If the energy production figures here reported are considered, we can estimate that MFC implemented in CWs the may cover between 3 and the 14% of the total energy demand.

> MFC implemented in CWs may cover between 3 and the 14 % of the total energy demand

CHAPTER 9

Conclusions

In this thesis the synergy between horizontal subsurface constructed wetlands (HSSF CW) and microbial fuel cells (MFC) was adressed. Initially, the operation of HSSF CW and the architecture and MFCs was reconsidered in order to obtain the maximum benefits from their synergy and an optimal solution was outlined. Then, CW-MFCs environmental applications were assessed. Electricity production, treatment efficiency improvement and organic pollution and clogging assessment were studied. The environmental and economic impacts of CW-MFCs were also determined.

In this chapter the reader will find the conclusions and some final remarks. The conclusions of the thesis are divided in 6 main blocks to facilitate the identification of each conclusion to the corresponding objective.

9.1 Conclusions

9.1.1 Optimal constructed wetland operational conditions

HSSF CWs constitute an optimal environment for MFCs implementation. Although the system is mainly anaerobic, the very upper part of the wetland remains under aerobic conditions due to its close contact with the atmosphere resulting in maximum redox gradients of 0.41 V vs SHE between the upper and the deeper layers. This natural redox gradient in HSSF CWs can be exploited to produce energy via MFC implementation.

The presence of macrophytes had an influence on the electrical output. However, this influence was, actually a consequence of water table variations which, in turn, were a consequence of evapotranspiration. Evapotranspiration caused cyclical water table and redox conditions at the cathode fluctuations and therefore, voltage generated by CW-MFCs followed the same cyclical daily pattern. As a consequence, the architecture of MFCs must cope with the effect of evapotranspiration caused by plants thus preventing the oxidized conditions at the cathode to vary along the day.

Anaerobic conditions both at the middle and the bottom layers of the wetland are fostered by a continuous hydraulic regime thus becoming the best hydraulic regime for MFC implementation when compared to the discontinuous flow regime. Furthermore, an anaerobic hydrolytic primary treatment (HUSB reactor) is the best option tested to be applied in a CW-MFC treatment plant. Under these conditions a higher predominance of bacteria from the Geobacteraceae family was found in anodic biofilms.

The relative predominance of the Geobacteraceae family in gravel and graphite samples of the HUSB line (19% and 5% of relative abundance for the graphite of active MFC and gravel samples, respectively) is also remarkable. Within the Geobacteraceae family the high relative abundance of one OTU belonging to *Geobacter* in active MFC of the HUSB line (from 13 to 16%) is of special interest. In the case of gravel and graphite samples from the settler line, Geobacteraceae were not only less favoured (below 2%), but even the detected Geobacteraceae OTUs were different from those found in samples from the HUSB line.

The OTU that was found to be the predominant species in anodic CW-MFC biofilms of the HUSB line was very similar in sequence to *Geobacter lovleyi*. *Geobacter lovleyi* showed a lack of electron donor or acceptor versatility which could introduce a source of limitation for CW-MFCs (neither lactate nor formate supported *G. lovleyi* growth). *G.lovleyi* required the presence of a strong reductant agent and the complete absence of oxygen to grow. This intolerance to oxygen could also become a limitation regarding CW-MFCs. Finally, *G. lovleyi* was demonstrated not to be very efficient at colonizing the graphite rod electrode.

9.1.2   Optimal MFC architecture

In CWs the real oxidized layer from which MFCs can benefit of very little extend and intense water table variations alter oxidized conditions at the cathode thus being cathode performance the major source of limitation for CW-MFCs. The optimal cathode position was found to be between 1 and 2 cm above water level. For all that, cell architecture shall address the possibility to cope with intense water level variations. To this regard, a cathode based on a thick layer of graphite placed at the upper part of the treatment bed will allow the CW-MFC to have always an active cathode zone for oxygen reduction, regardless the water level within the wetland. Due to the fact that water level can vary up 10 cm from the design water level, the thickness of the graphite layer shall be, at least, 10 cm. Also the distance between electrodes must be minimized in order to reduce the internal resistance of the system and given that all the bed is equally reduced, the anode can be placed as close as possible to the cathode but, also considering water level fluctuations, it must be kept deep enough to avoid its contact with the atmosphere.

The optimal cathode to anode surface ratio was determined to be between 3 and 4 cm^2/cm^2 under the conditions tested. At the beginning of its operation, when compared to anodic gravel-based CW-MFCs, graphite-based systems generated higher voltages and intensities. However, when biofilms were highly developed and systems were partially clogged, CW-MFCs working under different anode materials did not show any significant difference in performance. Finally, 220 ohms was proven to be the optimal external resistance for CW-MFCs treatment efficiency improvement.

9.1.3   CW's treatment efficiency improvement

HSSF CWs operated as MFCs were demonstrated to be a promising strategy to improve domestic wastewater treatment efficiency (either in terms of organic matter, ammonia and phosphates removal). The concentration of pollutants at the effluent of the non-connected systems was always statistically higher than at the connected CW-MFCs, indicating a stimulation of the organic matter oxidation as well as the ammonia and the phosphate removal in connected systems.

Gravel-based anode membrane-less MFCs operated at closed circuit showed ca. 21%, 18%, 15%, 31% and 25% lower effluent concentration than unconnected MFCs in regards to BOD_5, COD, TOC, PO_4^{-3} and NH_4^+-N, respectively.

9.1.4   CW-MFC for COD assessment

Positive linear relationships could be established between current generated and inlet COD concentration. This linearity was found both for gravel and graphite-based anode MFCs. Although, the best results were obtained after 20h of contact time, our biosensors showed correlations with R-square values higher than 0.8 also for contact times higher than 10 hours, regardless the material used. Graphite based MFCs showed a better biosensing capacity than gravel systems in terms of the correlation coefficients obtained and bioindication ranges.

Current originated from abiotic reactions at the anode accounted for less than the 2% of the biologically generated current regardless the anodic material considered.

MFC-based biosensors operated with real wastewater generate a background level current which comes both from the oxidation of the accumulated solids within the system and through endogenous metabolism which influences the bioindication capacity of the tool at the medium-long term.

Microbial fuel cells fed with wastewater and, especially, in the context of constructed wetlands, are subjected to very variable conditions which could

hinder MFCs bioindication capacity. Although results obtained in this work indicate that linear relationships can be established between inlet COD and current generated, there are several factors that can affect the precision, repeatability and operational stability of the devices. Therefore, other alternatives such as its utilization as a qualitative, range-like assessment tool should be considered for CW-MFCs rather than a specific-value assessment tool.

9.1.5   CW-MFC for clogging assessment

The sludge retained within the microbial fuel cells (MFC) had a direct effect on the MFC performance. Accordingly, the higher the sludge accumulated within the MFC the lower the amount of electrons transferred. The electric charge decreased as function of sludge retained following a potential equation. The accuracy of the MFC for clogging assessment was independent of the contact time considered.

After ca. 5 years of estimated clogging time the MFC was no longer able to produce any significant electric charge. This result suggests that, although longer study periods under more realistic conditions shall be further performed, MFC has great potential to be used as an indirect, continuous clogging assessment tool in constructed wetlands.

9.1.6   Environmental and economic impact of CW-MFCs

The CW systems coupled with MFCs are an appropriate solution for wastewater treatment in small communities which may help to reduce surface requirements, while keeping the environmental impacts low.

The CW system coupled with a graphite-based anode MFC appeared as the most environmentally friendly solution which could replace conventional CWs reducing system footprint by up to 20%.

The CW system coupled with a graphite-based anode MFC showed to be around 1.5 times more expensive than the conventional CW system. The cost of MFC-based CW would be competitive with conventional CW only

under higher treatment performances of MFC than those currently attained. For the purpose of reducing costs, cheaper electrode materials should be investigated for MFCs implementation in CW systems.

9.2 Final remarks

Thesis final remarks are the following:

- HSSF CWs constitute an optimal environment for MFCs implementation

- Continuous flow regime constitutes the best hydraulic regime for MFC implementation

- HUSB reactor was the best primary treatment to be applied in CW-MFCs. When it was implemented, the growth of bacteria from the Geobacteraceae family was fostered in anodic biofilms

- Although being a non-conductive material, gravel constitutes a suitable surface for bacteria from the Geobacteraceae family to stablish

- The architecture of MFCs must cope with the effect of evapotranspiration caused by plants thus preventing the variation of oxidized conditions at the cathode along the day

- Only when the cathode was around 1 to 2 cm above water level the cell voltage was maximized

- The anode must be placed as close as possible to the cathode but deep enough to avoid its contact with the atmosphere due to the evapotranspiration effect (from ca. 10 cm)

- Cathode to anode ratio of ca. 4 cm^2/cm^2 was enough to avoid cathodic limitation in CW-MFCs

- Solids accumulation within the anode might have caused a significant limitation in graphite-based systems to the point that no differences were seen between a graphite and gravel based CW-MFC performance

- 220 ohms was the optimal external resistance for CW-MFCs treatment efficiency improvement

- HSSF CWs operated as MFCs is a promising strategy to improve domestic wastewater treatment efficiency

- CW-MFCs are better suited as a qualitative, range-like semi-continuous COD assessment tool

- MFC has a great potential to be used as an indirect, continuous clogging assessment tool in constructed wetlands

- CW system coupled with graphite-based anode MFC appeared as the best alternative to reduce CW surface requirements (by around 20%) from an environmental perspective

- Although the high cost of materials (graphite and stainless-steel) used for MFCs implementation, CW systems coupled with high performance MFCs would be competitive with conventional CWs in terms of costs

- MFC implemented in CWs may cover between the 3 and the 14% of the total energy demand

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Supplementary material
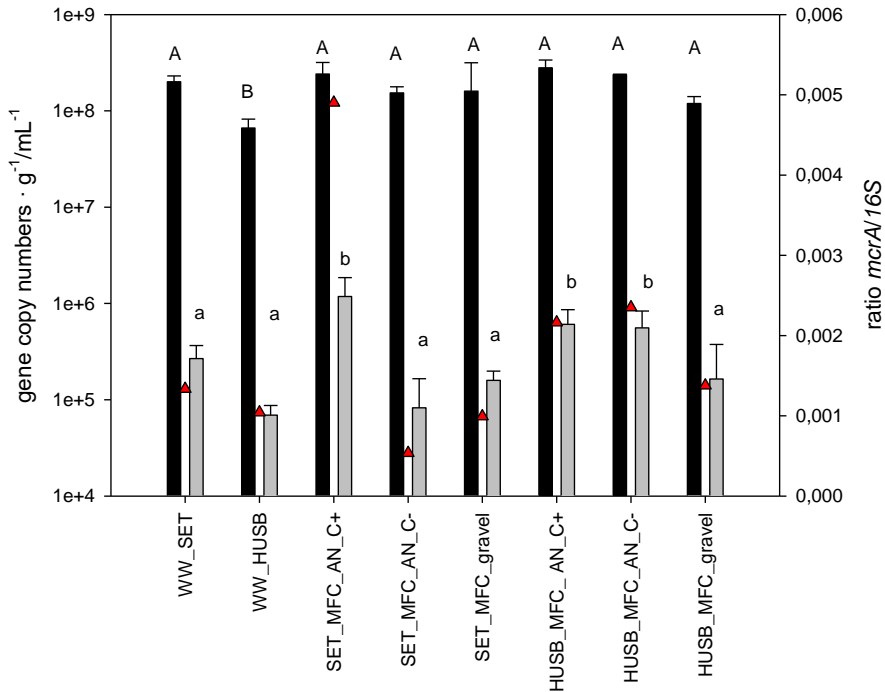
Figure 4.2.S1 Quantitative PCR analysis of *16S rRNA* and *mcrA* genes at different reactor configurations. Values are the average of triplicate qPCR reactions from three independent DNA extracts with standard deviations. Ratios *mcrA/16S rRNA* are displayed. WW: Wastewater; SET (Settler); HUSB; SET_MFC (settler line); HUSB_MFC (HUSB line) AN (graphite from anode); C+ (Closed circuit); C- (opened circuit); SET_MFC (MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (MFC implemented on constructed wetland running on wastewater from HUSB). Bars with the same letter style do not show significant (P> 0.05) differences according to the Tukey's HSD test. Black bar: *16S rRNA* gene; Grey bar: *mcrA* gene; Triangle: ratio *mcrA/16S rRNA* genes

Table 4.2.S1 Quantitative PCR analysis of *16S rRNA* and *mcrA* genes at different reactor configurations. Values are the average of triplicate qPCR reactions from independent DNA extracts with standard deviations. Ratios *mcrA/16S rRNA* are displayed. WW: Wastewater; SET (Settler); HUSB; SET_MFC (settler line); HUSB_MFC (HUSB line); AN (graphite from anode); C+ (closed circuit); C- (opened circuit); SET_MFC (MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (MFC implemented on constructed wetland running on wastewater from HUSB).

	16S rRNA gene	*mcrA* gene	*mcrA/16S* (%)
WW_SET	$2.01 \pm 0.30 \cdot 10^8$	$2.68E \pm 0.97 \cdot 10^5$	0.13%
WW_HUSB	$6.64 \pm 0.16 \cdot 10^7$	$6.92E \pm 0.18 \cdot 10^4$	0.10%
SET_MFC_AN_C+	$2.41 \pm 0.77 \cdot 10^8$	$1.18E \pm 0.67 \cdot 10^6$	0.49%
SET_MFC_AN_C-	$1.54 \pm 0.14 \cdot 10^8$	$8.26E \pm 8.2 \cdot 10^4$	0.05%
SET_MFC_gravel	$1.60 \pm 1.56 \cdot 10^8$	$1.59E \pm 0.39 \quad 10^5$	0.10%
HUSB_MFC_ AN_C+	$2.80 \pm 0.57 \cdot 10^8$	$6.07E \pm 2.54 \cdot 10^5$	0.22%
HUSB_MFC_AN_C-	$2.37 \pm 0.03 \cdot 10^8$	$5.58E \pm 2.75 \cdot 10^5$	0.24%
HUSB_MFC_gravel	$1.19 \pm 0.21 \cdot 10^8$	$1.65 \pm 2.11 \cdot 10^5$	0.14%
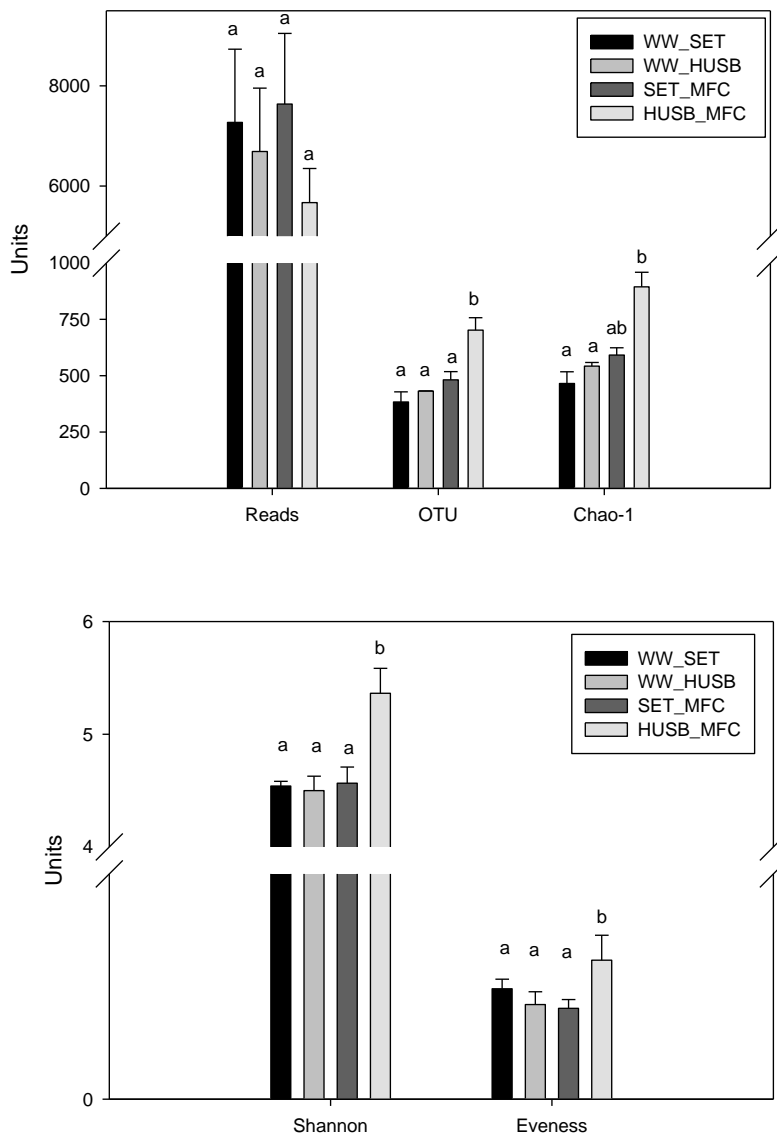
Figure 4.2.S2 Trimmed Reads, assigned OTUs, Chao-1, Evenness and Shannon-Wiener index represented as a mean value and standard deviation of wastewater samples (HUSB and settler), and MFC-implemented in wetland running either settler or HUSB pretreated wastewater. Bars with the same letter do not show any significant (P> 0.05) difference according to the Tukey's HSD test

Table 4.2.S2 Estimators of eubacterial (A) and archaeal (B) sequence libraries diversity, evenness and coverage. WW: Wastewater; SET (Settler); HUSB; AN (graphite from anode); C+ (Closed circuit); C- (opened circuit); SET_MFC (Settler line: MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (HUSB line: MFC implemented on constructed wetland running on wastewater from HUSB). Evenness was calculated using the formula (EXP(Shannon))/Sobs, in which Sobs is the observed number of species (OTUs). Good's estimator of coverage was calculated using the formula: $(1-(\text{singletons}/\text{individuals})) \times 100$. Estimators were calculated using EstimateS for each of the 16 clone libraries described in this study.

| | | | | | | EUBACTERIAL | | | |
| A) | | | | | | POPULATIONS | | | |
Sample	Description	Total reads	OTUs	Singletons	Chao 1	Shannon (Diversity)	Simpson Inv. (Diversity)	Coverage (%)	Evenness
M1736E1	WW_SET	6236	352	87	428±21	4,51	43,89	98,60	0,26
M1736E2		8304	415	102	502±23	4,57	42,73	98,77	0,23
M1740E1	WW_HUSB	7583	432	101	531±26	4,41	25,44	98,67	0,19
M1740E2		5794	431	114	554±31	4,59	32,5	98,03	0,23
M1737E1	SET_MFC_AN_C+	6652	489	128	610±28	4,74	33,68	98,08	0,23
M1737E2		6878	436	100	529±25	4,48	24,58	98,55	0,20
M1738E1	SET_MFC_AN_C-	6668	457	138	602±33	4,50	24,51	97,93	0,20
M1738E2		7329	492	126	594±24	4,62	27,88	98,28	0,21
M1739E1	SET_MFC_Gravel	7968	472	128	591±28	4,36	21,28	98,39	0,17
M1739E2		10330	543	117	622±19	4,69	32,74	98,87	0,20
M1741E1	HUSB_MFC_AN_C+	5500	640	202	837±37	5,00	28,7	96,33	0,23
M1741E2		5140	664	213	865±38	5,19	38,33	95,86	0,27
M1742E1	HUSB_MFC_AN_C-	5020	693	217	916±40	5,49	79,85	95,68	0,35
M1742E2		5315	676	189	821±28	5,54	96,59	96,44	0,38
M1743E1	HUSB_MFC_Gravel	6667	765	217	942±32	5,42	57,87	96,75	0,29
M1743E2		6363	775	227	986±37	5,54	75,87	96,43	0,33

					ARCHAEAL POPULATIONS				
B)									
Samples	Description	Total reads	OTUs	Singletons	Chao 1	Shannon (Diversity)	Simpson Inv. (Diversity)	Coverage (%)	Evenness
M1736E1	WW_SET	172	14	4	16±3	2,01	5,90	97,67	0,53
M1736E2		152	16	3	17±1	1,95	4,54	98,03	0,44
M1740E1	WW_HUSB	188	14	5	16±3	1,27	2,07	97,34	0,25
M1740E2		653	22	2	22±1	1,63	2,42	99,69	0,23
M1737E1	SET_MFC_AN_C+	633	26	5	31±6	1,93	3,77	99,21	0,27
M1737E21		74	11	4	13±3	1,84	4,85	94,59	0,57
M1738E1	SET_MFC_AN_C-	41	7	0	7±1	1,75	4,93	100,00	0,82
M1738E2		33	8	1	8±0	1,87	5,64	96,97	0,81
M1741E1	HUSB_MFC_AN_C+	572	30	9	48±16	1,87	3,16	98,43	0,22
M1741E2		1187	37	7	44±7	2,14	4,69	99,41	0,23
M1742E1	HUSB_MFC_AN_C-	2615	48	11	56±6	1,54	2,10	99,58	0,10
M1742E2		2612	49	9	52±3	1,87	3,22	99,66	0,13
M1743E1	HUSB_MFC_Gravel	1786	45	11	56±9	2,18	5,18	99,38	0,20
M1743E2		2157	49	11	56±6	2,09	4,11	99,49	0,16

Table 4.2.S3-A Biodiversity of eubacteria (class) (A) and archaea (family) (B) expressed as relative OTUs abundance (%), in the treated wastewater and the different reactor setups. WW: Wastewater; SET (Settler); HUSB; AN (graphite from anode); C+ (Closed circuit); C- (opened circuit); SET_MFC (Settler line: MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (HUSB line: MFC implemented on constructed wetland running on wastewater from HUSB).

| | | WASTEWATER | | | | SETTLER LINE | | | | | | HUSB LINE | | | | | |
| | | settler | | HUSB | | C+ | | C- | | gravel | | C+ | | C- | | gravel | |
		AV	SD	AV	SD	AVG	STD	AVG	STD	AVG	STD	AVG	STD	AVG	STD	AVG	STD
Eubacteria	Alphaproteobacteria	1,4	0,0	1,8	0,1	32,9	0,3	31,5	1,2	25,2	5,8	13,4	1,5	14,6	0,4	17,0	1,3
	Betaproteobacteria	28,9	1,3	46,1	0,6	12,8	2,4	13,4	2,5	16,0	1,6	3,0	0,1	3,4	0,7	3,4	0,3
	Gammaproteobacteria	12,3	0,5	31,5	5,7	6,9	0,8	8,6	1,3	17,0	10,2	1,1	0,2	0,9	0,0	0,9	0,0
	Deltaproteobacteria	0,0	0,0	0,1	0,0	2,1	0,0	2,0	0,6	2,9	1,7	30,6	2,6	16,8	0,2	16,8	3,0
	clostridia	2,0	1,0	6,5	2,6	3,0	0,1	1,9	0,8	1,7	0,5	12,9	2,4	18,2	0,7	14,8	1,6
	Bacterioidia	5,3	0,3	3,2	0,5	0,3	0,0	0,4	0,0	0,4	0,0	5,6	0,4	7,6	1,2	6,6	0,7
	Synergistia	0,1	0,1	0,7	0,3	0,0	0,0	0,0	0,0	0,0	0,0	8,4	0,5	9,2	0,3	8,5	0,2
	Flavobacteria	47,9	0,5	6,3	0,6	25,5	6,4	28,7	3,0	21,2	6,0	0,7	0,2	0,7	0,0	0,7	0,0
	Holophagae	0,0	0,0	0,1	0,0	0,0	0,0	0,7	0,6	3,9	4,5	0,6	0,3	1,1	0,2	1,1	0,1
	Anaerolineae	0,1	0,0	0,2	0,1	0,2	0,2	0,1	0,1	0,1	0,0	5,2	0,9	6,0	0,6	4,4	0,8
	Other	1,9	0,0	3,7	0,8	16,2	3,1	12,6	1,3	11,6	2,6	18,4	2,9	21,5	0,2	25,8	3,1

Table 4.2.S3-B Biodiversity of eubacteria (class) (A) and archaea (family) (B) expressed as relative OTUs abundance (%), in the treated wastewater and the different reactor setups. WW: Wastewater; SET (Settler); HUSB; AN (graphite from anode); C+ (Closed circuit); C- (opened circuit); SET_MFC (Settler line: MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (HUSB line: MFC implemented on constructed wetland running on wastewater from HUSB).

		WASTEWATER				SETTLER LINE						HUSB LINE					
		settler		HUSB		C+		C-		grav		C+		C-		grav	
		AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD
Archaea	Methanosaetaceae	55,0	4,9	81,3	4,7	4,0	1,2	0,0	0,0	-	-	17,6	1,9	3,8	0,1	11,2	1,2
	Methanobacteriaceae	29,0	16,9	13,1	0,0	2,3	1,0	33,2	10,5	-	-	0,6	0,4	0,0	0,0	0,3	0,3
	Methanomicrobiaceae/ Thermoplasmata	13,1	15,3	1,4	2,0	15,2	21,4	4,7	6,6	-	-	56,9	11,7	70,6	14,2	47,8	7,0
	Methanospirillaceae	0,0	0,0	3,3	1,4	0,0	0,0	0,0	0,0	-	-	0,8	0,3	0,5	0,1	1,0	0,5
	Methanosarcinaceae	3,2	3,7	0,0	0,0	25,3	28,3	25,7	36,3	-	-	1,4	0,4	0,4	0,1	0,7	0,3
	Fervidicoccaceae/ Thermoprotei	0,0	0,0	0,0	0,0	53,4	4,7	36,5	19,2	-	-	22,8	9,5	24,7	14,4	39,1	8,3
	Thermoplasmataceae	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	-	-	0,0	0,0	0,1	0,1	0,1	0,1
	Nitrososphaeraceae	0,0	0,0	0,9	1,2	0,0	0,0	0,0	0,0	-	-	0,0	0,0	0,0	0,0	0,0	0,0

Table 4.2.S4 Biodiversity of eubacteria (phylum) expressed as relative OTUs abundance (%), in the treated wastewater and the different reactor setups. WW: Wastewater; SET (Settler); HUSB; AN (graphite from anode); C+ (Closed circuit); C- (opened circuit); SET_MFC (Settler line: MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (HUSB line: MFC implemented on constructed wetland running on wastewater from HUSB).

| Phylum | wastewater | | | | SETTLER | | | | | | HUSB | | | | | |
| | settler | | HUSB | | C+ | | C- | | gravel | | C+ | | C- | | gravel | |
	AV	STD	AV	STD	AVG	STD	AVG	STD	AVG	STD	AVG	STD	AVG	STD	AVG	STD
tm7 (candidate division)	0,2	0,1	0,0	0,0	4,5	0,1	2,9	0,4	3,8	0,5	1,0	0,4	0,7	0,1	1,2	0,3
actinobacteria	0,8	0,1	0,9	0,4	6,2	1,8	6,3	2,1	5,5	3,3	5,0	0,3	4,0	0,3	6,3	1,5
synergistetes	0,1	0,1	0,7	0,3	0,0	0,0	0,0	0,0	0,0	0,0	8,5	0,5	9,2	0,4	8,6	0,2
planctomycetes	0,0	0,0	0,1	0,1	0,4	0,1	0,3	0,1	0,2	0,2	0,6	0,2	1,3	0,1	1,2	0,1
op3 (candidate division)	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,7	0,4	2,0	0,1	1,1	0,1
proteobacteria	43,0	1,8	80,3	4,7	55,1	2,9	55,8	1,7	61,6	4,9	48,7	4,2	36,3	0,1	38,9	4,7
tenericutes	0,0	0,0	0,2	0,1	0,0	0,0	0,1	0,1	0,0	0,0	0,3	0,1	0,7	0,1	0,7	0,1
nitrospirae	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,7	0,1	2,8	1,2	2,3	0,1
firmicutes	2,4	1,0	7,5	2,8	3,2	0,2	2,4	0,4	1,9	0,6	14,9	2,3	20,4	0,3	18,1	1,6
bacteroidetes	53,5	0,8	9,6	1,3	29,8	5,8	30,8	4,5	22,4	5,7	8,4	0,5	10,8	0,1	9,9	0,6
acidobacteria	0,0	0,0	0,1	0,1	0,3	0,2	0,8	0,6	4,0	4,5	0,8	0,4	1,4	0,1	1,6	0,1
spirochaetes	0,0	0,0	0,1	0,1	0,0	0,0	0,2	0,1	0,1	0,1	0,4	0,1	0,7	0,1	1,1	0,0
chloroflexi	0,1	0,0	0,2	0,1	0,5	0,4	0,3	0,1	0,4	0,1	6,5	0,6	7,0	0,8	6,6	1,7

Table 4.2.S5 Geobacteriaceae OTUs distribution expressed as relative OTUs abundance (%) in the treated wastewater and the different reactor setups. WW: Wastewater; SET (Settler); HUSB; AN (graphite from anode); C+ (Closed circuit); C- (opened circuit); SET_MFC (Settler line: MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (HUSB line: MFC implemented on constructed wetland running on wastewater from HUSB).

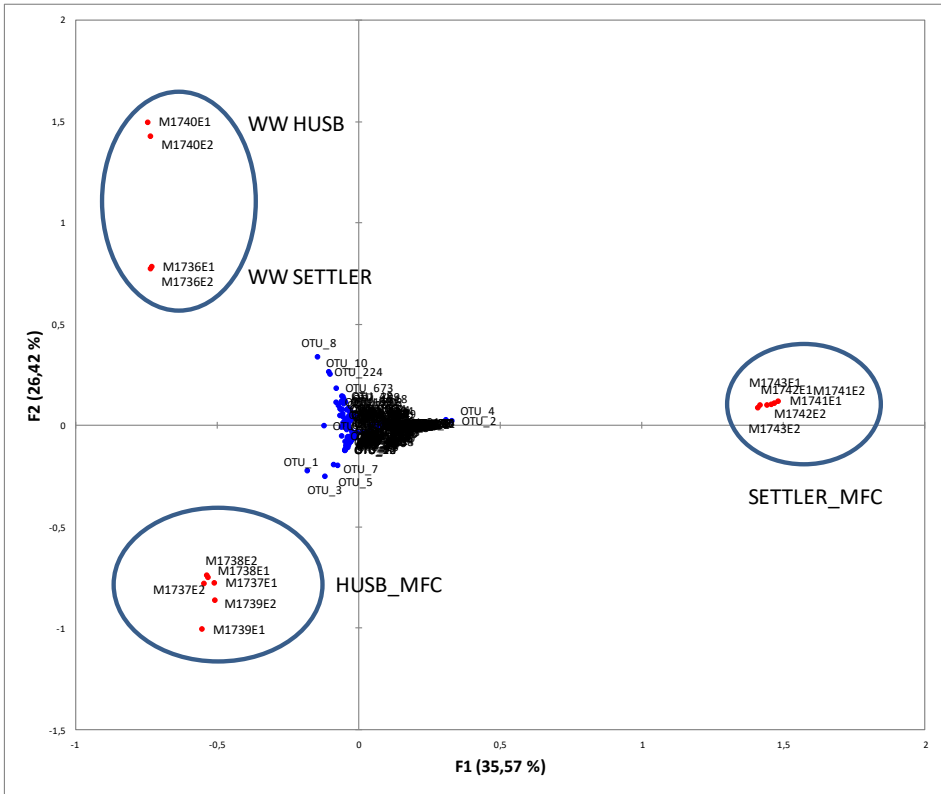| OTU name	wastewater				SETTLER						HUSB					
	settler		HUSB		C+		C-		gravel		C+		C-		gravel	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4	0	0	0	0	0	0	0	0	0	0	15,9	13,3	0,6	1,5	3,7	3,4
46	0	0	0	0	0	0,1	0,1	0,5	2	0,3	0	0	0	0	0	0,1
47	0	0	0	0	0	0	0	0	0	0	0,8	0,6	1	1,4	0,3	0,4
209	0	0	0	0	0	0	0	0	0	0	0,1	0	0,3	0,5		0,1
283	0	0	0	0	0	0	0	0	0,2	0	0	0,1	0	0,1	0	0
398	0	0	0	0	0,1	0,2	0	0	0	0	0	0	0	0	0	0
504	0	0	0	0	0	0	0	0	0	0	0,1	0,2	0	0	0	0
509	0	0	0	0	0	0	0	0	0	0,1	0	0	0	0	0	0
526	0	0	0	0	0	0,1	0	0,1	0,8	0,1	0	0	0,1	0	0	0,1
650	0	0	0	0	0	0	0	0	0	0	0,2	0	0	0	0	0
654	0	0	0	0	0	0	0	0	0	0	0	0	0,1	0,1	0	0
787	0	0	0	0	0	0	0	0	0	0	0,4	0,4	0	0	0,1	0,1
1207	0	0	0	0	0	0	0	0	0	0	0	0	0,1	0	0	0
1328	0	0	0	0	0	0	0	0	0	0	0	0	0,1	0	0	0
1338	0	0	0	0	0	0	0	0	0	0	0,1	0	0	0	0	0
1425	0	0	0	0	0	0	0	0	0	0	0,1	0	0	0	0	0
1640	0	0	0	0	0	0	0	0,1	0	0	0,1	0	0	0	0	0
1758	0	0	0	0	0	0	0	0	0	0	0,1	0	0	0	0	0
1950	0	0	0	0	0	0	0	0	0	0	0,3	0,2	0	0	0,1	0,1
1972	0	0	0	0	0	0	0	0	0	0	0,1	0	0	0	0	0
1990	0	0	0	0	0	0	0	0	0,1	0	0	0	0	0	0	0
2061	0	0	0	0	0	0	0	0	0	0	0	0,1	0	0	0	0
2067	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2082	0	0	0	0	0	0	0	0	0	0	2,3	2	0,1	0,2	0,4	0,2
2088	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOT	0	0	0	0	0,1	0,4	0,1	0,7	3,1	0,5	20,6	16,9	2,4	3,8	4,6	4,5

Figure 4.2.S3 Correspondence analysis (CA) biplot of eubacterial OTUs distribution from pyrosequencing analysis implemented on different reactor setups. WW: Wastewater; SET (Settler); HUSB; AN (graphite from anode); C+ (Closed circuit); C- (opened circuit); SET_MFC (Settler line: MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (HUSB line: MFC implemented on constructed wetland running on wastewater from HUSB)
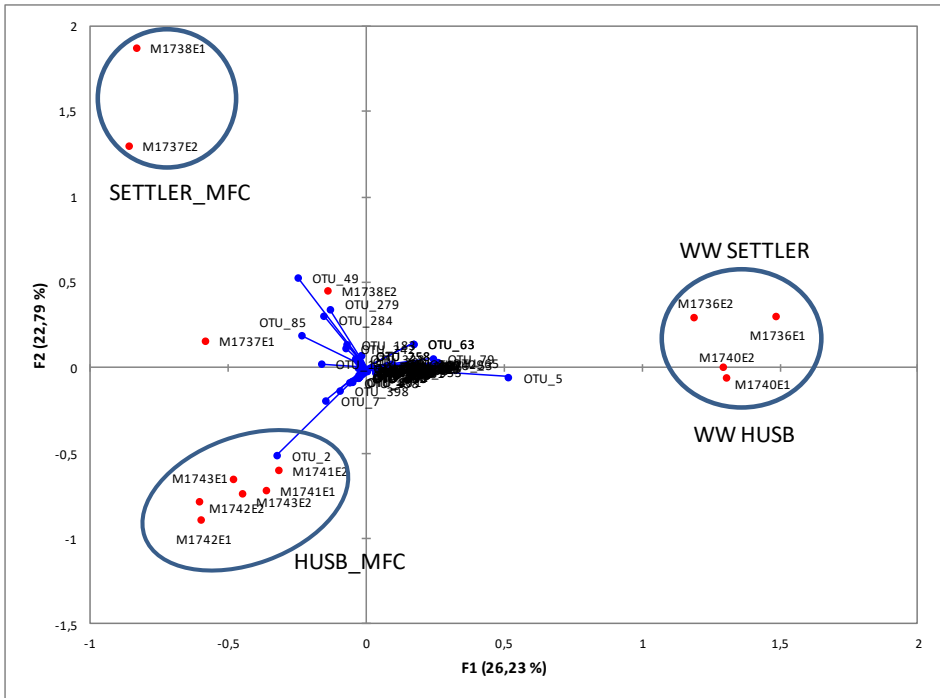
Figure 4.2.S4 Correspondence analysis (CA) biplot of archaeal OTUs distribution from pyrosequencing analysis implemented on different reactor setups. WW: Wastewater; SET (Settler); HUSB; AN (graphite from anode); C+ (Closed circuit); C- (opened circuit); SET_MFC (Settler line: MFC implemented on constructed wetland running on wastewater from settler); HUSB_MFC (HUSB line: MFC implemented on constructed wetland running on wastewater from HUSB).
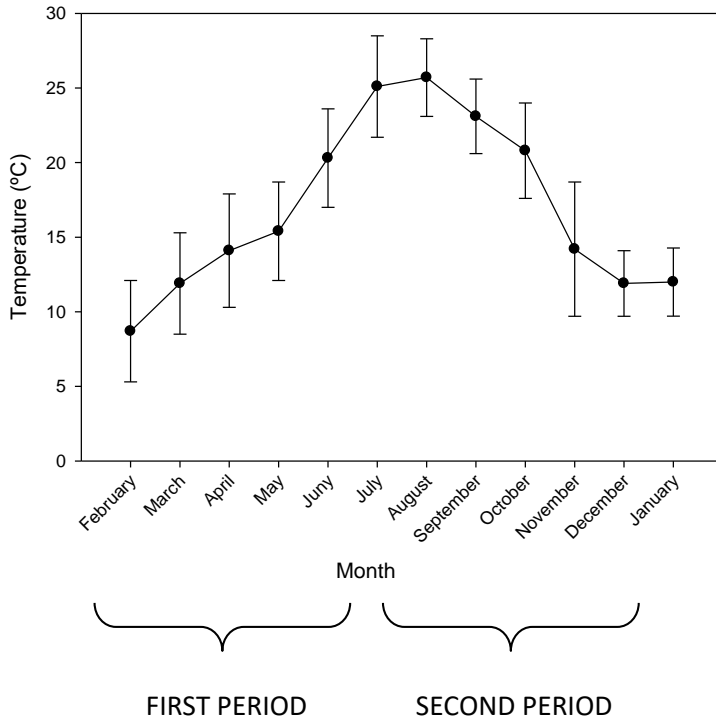
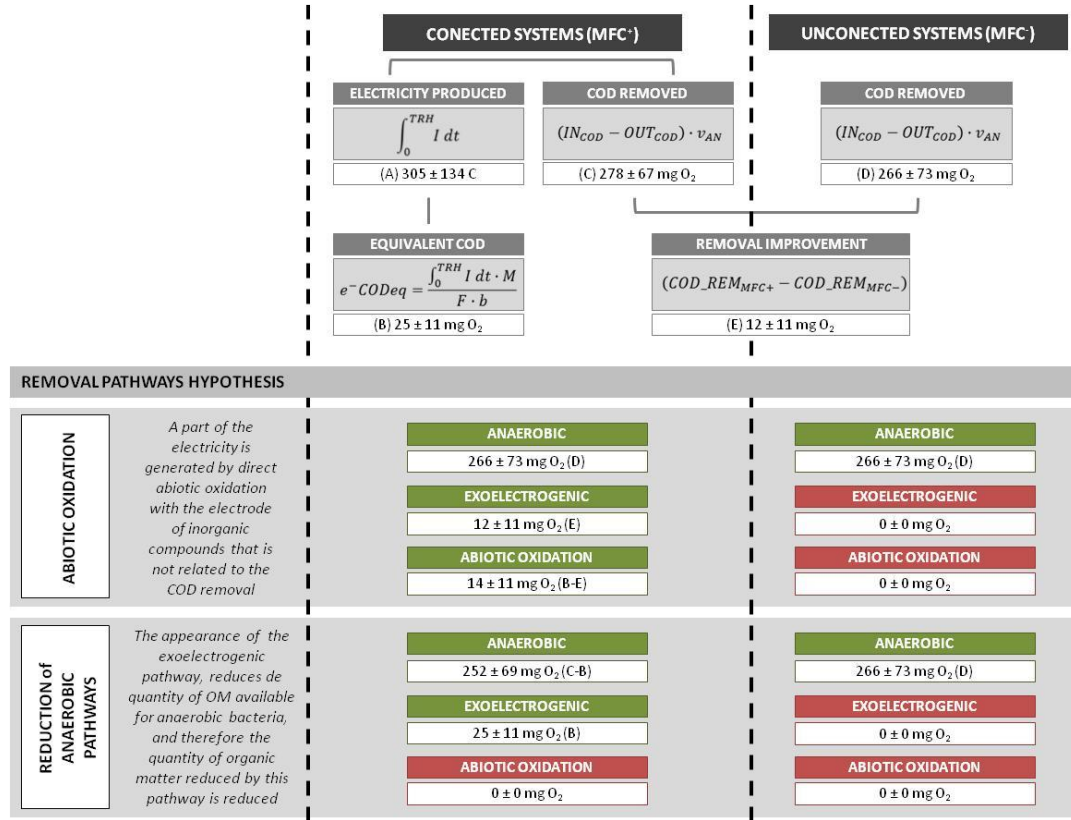**Figure 4.3.S1** Average monthly temperatures

CHAPTER 5

Figure 5.S1 Outline of the calculation of the equivalent organic matter removed in active and non-active systems and the envisaged hypothesis for encountered differences between MFC+ and MFC-
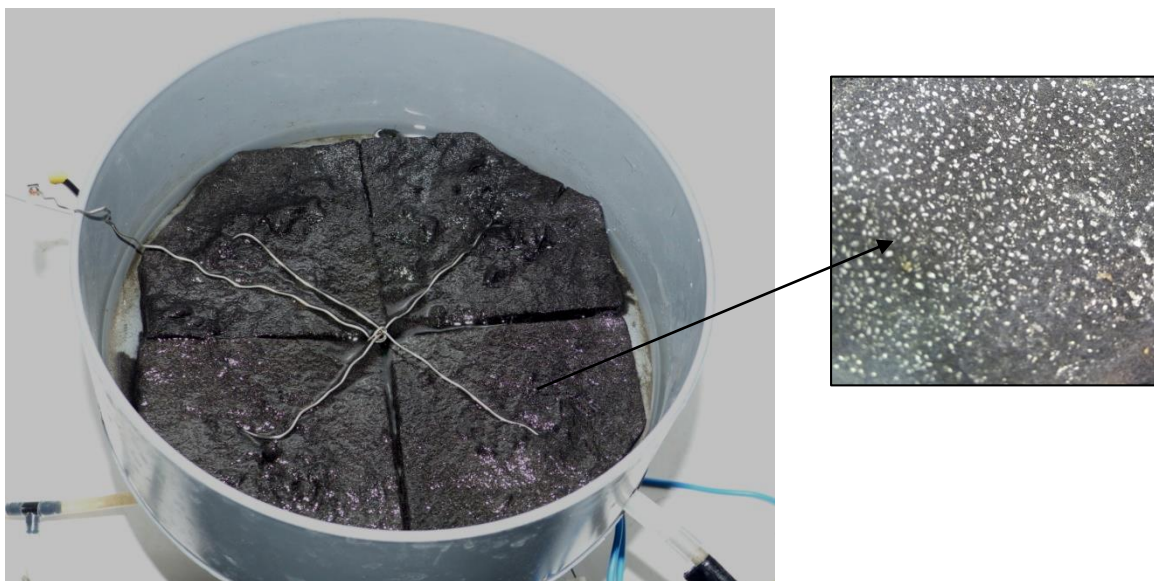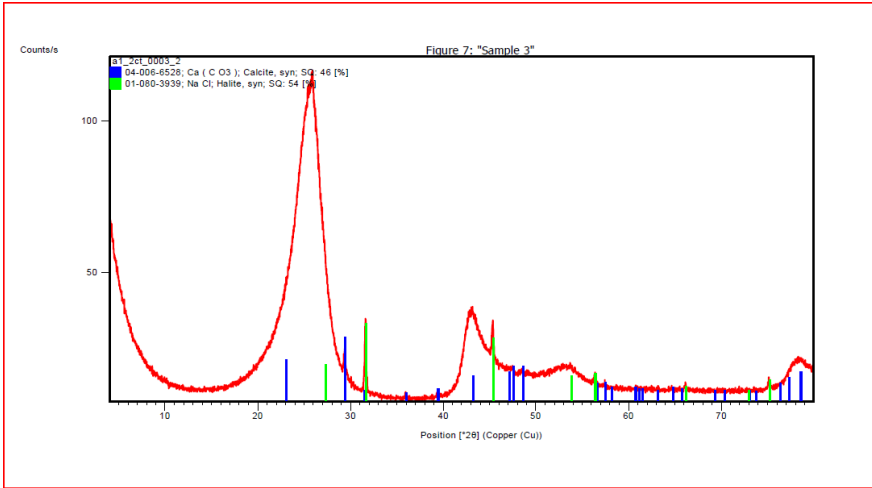
Figure 5.S2 Precipitates around the cathode of active CW-MFC

A)

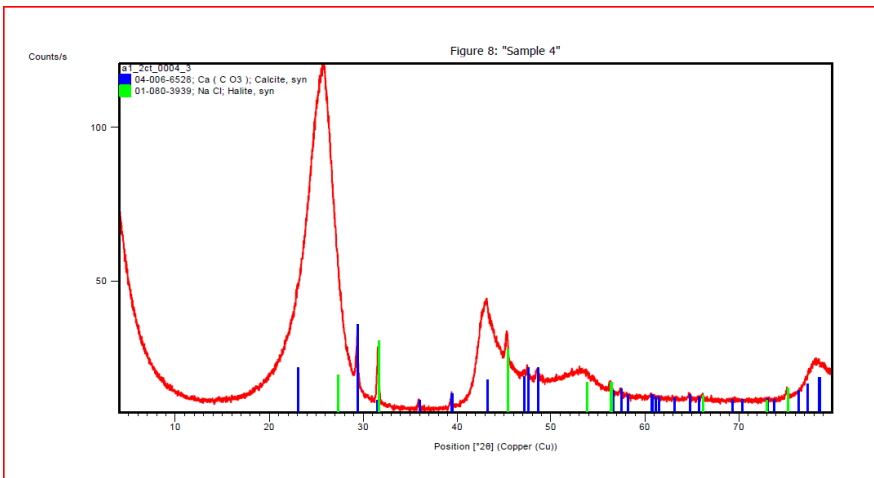

B)

Figure 5.S3 A: example of x-ray diffractogram from gravel-based MFC⁺; B: example of x-ray diffractogram from gravel-based MFC⁻
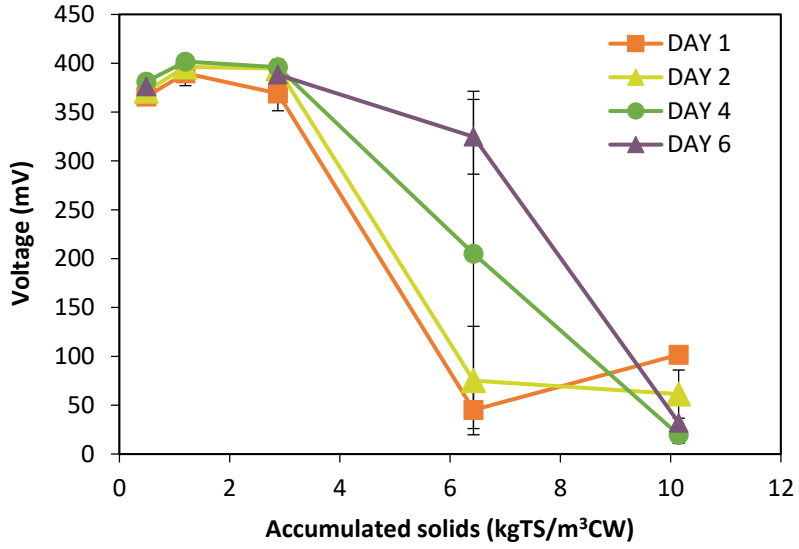
Fig 6.2.S1 Voltage generated against accumulated solids at different contact times (1, 2, 4 and 6 days)

Agraïments

Vull destinar unes línies d'agraïment a totes aquelles persones que han contribuït en la realització d'aquesta tesi. Sense elles no hauria estat possible.

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Catalunya per haver-me concedit els ajuts que m'han permès dur a terme els estudis de doctorat.

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Curriculum Vitae

Clara Corbella was born in Barcelona in 1987. She obtained her bachelor degree in Civil Engineering from the Universitat Politècnica de Catalunya (UPC) in 2012. In 2013 she started her PhD at the Group of Environmental Engineering and Microbiology (GEMMA) in the UPC.

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EDUCATION

2013 to date	**PhD student in Environmental Engineering Program** Universitat Politècnica de Catalunya – BarcelonaTech
April - September 2015	**Stay at Michigan State University (USA)** Department of Microbiology and Molecular Genetics Supervisor: Gemma Reguera Topics: Cell growth limitations in MFCs
2005 - 2012	**BSc and MSc in Civil Engineering** Civil Engineering School of Barcelona (ETSECCPB) Universitat Politècnica de Catalunya – BarcelonaTech *Master thesis: Greenhouse gases emission in constructed wetlands: Adaptation of the closed chamber method and measurements as function of the hydraulic regime and the presence of macrophytes*

LANGUAGE SKILLS

English	Full professional proficiency (C1.2 - European Framework of Reference)
Spanish	Native or bilingual proficiency.
Catalan	Native or bilingual proficiency.

2017   **Corbella, C.** and Puigagut, J. Novel membrane-less MFC-based biosensor for domestic wastewater COD assessment in constructed wetlands. In preparation.

2017   **Corbella, C.,** Puigagut, J. Improving domestic wastewater treatment efficiency with constructed wetland microbial fuel cells: influence of anode material and external resistance. In preparation.

2017   **Corbella, C.**, Steidl, R. P., Puigagut, J., and Reguera, G. Electrochemical characterization of *Geobacter lovleyi* identifies limitations of microbial fuel cell performance in constructed wetlands. International Microbiology. 20(2) 55–64.

2017   **Corbella, C.**, Puigagut, J., Garfí, M. Life cycle assessment of constructed wetland systems for wastewater treatment coupled with microbial fuel cells. Science of the Total Environment. 584–585, 355–362.

2016   **Corbella, C.**, García, J. i Puigagut, J. Microbial fuel cells for clogging assessment in constructed wetlands. Science of the Total Environment. 569-570 1060-1063

2016   **Corbella, C.**, Garfí, M. Puigagut, J. Long-term assessment of best cathode position to maximize microbial fuel cell performance in horizontal subsurface flow constructed wetlands. Science of the Total Environment. 563–564 448–455

2015   **Corbella, C.**, Guivernau, M., Viñas, M, Puigagut, J. Power production with microbial fuel cells implemented in constructed wetlands: operational, design and microbial aspects. Water Research (84) 232-242.

2015   **Corbella, C.**, Puigagut, J. Effect of primary treatment and organic loading on methane emissions from horizontal subsurface flow constructed wetlands treating urban wastewater. Ecological Engineering (80) 79-84

2014   **Corbella, C.**, Garfí. A. and Puigagut, J. Vertical redox profiles in treatment wetlands as function of hydraulic regime and macrophytes presence: Surveying the optimal scenario for microbial fuel cell implementation. Science of the Total Environment. 470-471, 754-758.

| 2013 | **Corbella, C.** and Puigagut, J. Improving the reliability of closed chamber methodologies for methane emissions measurement in treatment wetlands. Water Science and Technology. 68(9) 2097-2102 |

OTHER PUBLICATIONS

| 2015 | **Corbella, C.** and Puigagut, J. Microbial Fuel Cells implemented in constructed wetlands: Fundamentals, current research and future perspectives. Contributions to Science.11:113-120 |

INVITED ORAL PRESENTATIONS

| 2015 | **Corbella, C.** Women in the academic ladder. Institut d'Estudis Catalans. Barcelona. |
| 2014 | **Corbella, C.** and Puigagut, J. Microbial Fuel Cells in Constructed Wetlands: challenges and opportunities. 3rd Low Energy Wastewater Treatment System Conference. Cranfield University. |

PARTICIPATION IN RESEARCH PROJECTS

| 2011-2014 | ENERWET: Implementation of Microbial Fuel Cells in constructed wetlands in order to optimize the removal efficiency, to generate electricity and to reduce greenhouse gases emission. Financed by Spanish Ministry of Science and Innovation (Plan Nacional de I+D+i, MICINN). Coordinated by the Group of Environmental Engineering and Microbiology. |
| 2011 | NAWATECH: Natural Water Systems and Treatment Technologies to cope with Water Shortages in Urbanized Areas in India. Financed by 7th Framework Program of the European Union (EU FP7). |

2016   **Corbella, C.** i Puigagut, J. Constructed Wetland-Microbial Fuel Cell enhances domestic wastewater treatment efficiency. 3rd International Meeting of the International Society for Microbial and Electrochemistry Technology (EU-ISMET). Roma, Italy. Oral communication.

2016   **Corbella, C.**, Solé, M., Arias, D., Solimeno, A., Uggetti, E., Garfí, M., Puigagut, J., Ferrer I., García, J. Líneas de investigación del Grupo de Ingeniería y Microbiología del Medio Ambiente (GEMMA). Mesa Española de Tratamiento de Aguas 2016 (META). Madrid, Espanya. Poster presentation.

2016   **Corbella, C.** i Puigagut, J. Pilas de combustible microbianas implementadas en humedales construidos. Española de Tratamiento de Aguas 2016 (META). Madrid, Espanya. Oral presentation.

2016   **Corbella, C.** i Puigagut, J. Microbial Fuel Cells implemented in Constructed Wetlands. Xth International Society of Environmental Biotechnology (ISEB). Barcelona, Espanya. Oral presentation.

2015   **Corbella, C.,** Gómez, N. and Puigagut, J. Novel microbial fuel cell-based biosensor for the assessment of organic loading in constructed wetlands. Wetland Pollutant Dynamics and Control (WETPOL) York (UK).

2015   **Corbella, C.** and Puigagut, J. Microbial fuel cells to improve treatment efficiency in constructed wetlands. Wetland Pollutant Dynamics and Control (WETPOL) York (UK).

2014   **Corbella, C.** and Puigagut, J. Influence of water level variation within constructed wetlands on power production with microbial fuel cells. 14th International Conference on Wetland Systems for Water Pollution Control (IWA). Shanghai, Xina. Oral Presentation.

2014   **Corbella, C.**, Guivernau, M., Viñas, M. and Puigagut, J. Effect of primary treatment on electrogenic bacteria populations colonizing the electrodes of microbial fuel cells implemented in constructed wetlands. 14th International Conference on Wetland Systems for Water Pollution Control (IWA). Shanghai, Xina. Oral presentation.

2014 **Corbella, C.,** Puigagut, J. Influence of cathode to anode surface ration on the electrical output generated by MFCs implemented in constructed wetlands during the treatment of domestic wastewater. 2nd European meeting of the International Society for Microbial Electrochemistry and Technology. Alcalá de Henares. Poster presentation.

2014 **Corbella, C.,** Puigagut, J. Contribution of macrophytes to the electrical output generated by MFCs implemented in constructed wetlands during the treatment of domestic wastewater. 2nd European meeting of the International Society for Microbial Electrochemistry and Technology. Alcalá de Henares. Poster.

2013 **Corbella, C.** and Puigagut, J. Energy production with microbial fuel cells implemented in horizontal subsurface flow treatment wetlands. 5th International Symposium on Wetland Pollutant Dynamics and Control. Nantes, France. Oral presentation.

2013 **Corbella, C.** and Puigagut, J. "Preliminary results on methane emission from horizontal subsurface flow treatment wetlands as function of primary treatment". 5th International Symposium on Wetland Pollutant Dynamics and Control. Nantes, France. Poster and Oral presentation.

2012 Garfi, A., **Corbella, C.** and Puigagut, J. "Surveying the best redox gradient scenario in horizontal subsurface flow treatment wetlands for optimal microbial fuel cells implementation". 13th International Conference Wetlands Systems for Water Pollution Control. (IWA specialist conferences). Perth, Australia. Oral presentation.

2012 **Corbella, C**. and Puigagut, J. "Improving the reliability of closed chamber methodologies for methane emissions measurement in treatment wetlands". 13th International Conference Wetlands Systems for Water Pollution Control (IWA specialist conferences). Perth, Australia. Poster.

2016-2017	Life Cycle Assessment of CW intensification with MFCs. Master Thesis. Student: Neus Montero Vilar.
2015-2016	Determination of the biotic and abiotic component of the MFC electric current in CW-MFCs. Master Thesis. Student: Claudia Jimenez Sarrión.
2015-2016	Membrane-less microbial fuel cell for semi-continuous assessment of treatment efficiency in pilot-scale constructed wetlands. Master Thesis. Student: Manel Mateu Casan.
2015-2016	Determination of the clogging state of constructed wetlands by means of CW-MFCS. Master Thesis. Student: Marc Fortet Fontseca.
2014-2015	Influence of cathode to anode distance on power output from Microbial Fuel Cells implemented in Constructed Wetlands: laboratory and pilot scale. Master Thesis. Student: Laura Martínez Crespo.
2014-2015	Microbial Fuel Cells implemented in Constructed Wetlands: biosensing capacity. Master Thesis. Student: Noelia Gómez Lafuente.
2014-2015	Influence of macrophytes, cathode to anode ratio and distance on power output from Microbial Fuel Cells implemented in Constructed Wetlands. Master Thesis. Student: Josep Yáñez de Ugarte.
2014-2015	Tratamiento de aguas residuals mediante digestor HUSB. Master Thesis. Student: Julio Alejandro Ríos Hernández.
2013-2014	Influence of aeration on Microbial Fuel Cells implemented in Constructed Wetlands. Master Thesis. Student: Neftalí Cruces Delgado.
2013-2014	Influence of organic loading on methane emissions from Horizontal Subsurface Constructed Wetlands. Master Thesis. Student: Mònica Sampé Cardelús.
2013-2014	Horizontal Subsurface Constructed Wetlands: determination of the optimal primary treatment. Master Thesis. Student: Alberto Ayllón Pérez.
2013-2014	Microbial Fuel Cells implemented in Constructed Wetlands: influence of cathode position. Master Thesis. Student: Juan José Cantero Deus.
2013-2014	Optimization of electrical power from Microbial Fuel Cells implemented in Constructed Wetlands. Master Thesis. Student: Aitor Álava Ordás.

2013-2014	Microbial fuel cells implemented in constructed wetlands for the treatment of domestic wastewater: effect of the anodic material on electrical production. Master thesis. Student: Cristina Torras Velasco.
2012-2013	Microbial fuel cells implemented in constructed wetlands for the treatment of domestic wastewater: influence of primary treatment. Universitat Politècnica de Catalunya. Master Thesis. Student: Antonio Perelló Moragues.
2012-2013	Microbial fuel cells implemented in constructed wetlands for the treatment of domestic wastewater: influence of organic loading. Universitat Politècnica de Catalunya. Master Thesis. Student: Elisabet Sorigué Bosch.

TEACHING EXPERIENCE

February-May 2015, 2016	Environmental engineering laboratory, Environmental Engineering Master. DECA (UPC). Theory and practice about drinking water and wastewater treatment. 30 hours
February 2016	Environmental systems, Environmental Engineering Master. DECA (UPC). Environmental bioindication class. 6 hours.
January-February 2014, 2015, 2016	"Water, how do we clean it?" UPC. Theory and practice about drinking water treatment. 20 hours.

OTHER ACHIEVEMENTS

2016	Best Oral Presentation in 10th ISEB Conference (Barcelona)
2016	Organizing committee of 10th ISEB Conference (Barcelona)
2014	Award given to students by the Catalan Society of Technology (Institut d'Estudis Catalans).
2014	Research fellowship granted by Generalitat de Catalunya (Ajut per a la contractació de personal investigador novell, FI-2014 AGAUR). RESOLUCIÓ ECO/1639/2013, de 22 de juliol de 2013.
2013	Poster award in 5th International Symposium on Wetland Pollutant Dynamics and Control. Nantes, France.