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

Article Type: Research Paper

Keywords: constructed wetlands; domestic wastewater; microbial fuel cells; Geobacter

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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 was profiled on wetland gravel, MFC electrodes and primary treated wastewater by means of 16S rRNA gene-based 454-pyrosequencing and qPCR of 16S rRNA and mcrA genes. Maximum current (219 mA/m²) and power (36 mW/m²) 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, MFCs show promise for power production in constructed wetlands receiving the effluent of a HUSB reactor.

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Highlights

- Cell voltage followed a daily pattern consistent with water level variation.
- Maximum current and power densities were that of 219 mA/m² and 36 mW/m².
- The primary treatment affected the diversity of bacteria colonizing MFCs.
- The primary treatment affected the relative abundance of bacteria colonizing MFCs.
- A high predominance of one OTU belonging to *Geobacter* was found in anodes biofilm.
CONSTRUCTED WETLAND TREATMENT PLANT

1. Homogenization tank; 2. HUSB reactor; 3. Settlers; 4 and 5. Horizontal Subsurface Constructed Wetlands

CONSTRUCTED WETLAND MICROBIAL FUEL CELLS

Graphical Abstract (for review)
Operational, design and microbial aspects related to power production with microbial fuel cells implemented in constructed wetlands

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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 was profiled on wetland gravel, MFC electrodes and primary treated wastewater by means of 16S rRNA gene-based 454-pyrosequencing and qPCR of 16S rRNA and mcrA genes. Maximum current (219 mA/m²) and power (36 mW/m²) 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, MFCs show promise for power production in constructed wetlands receiving the effluent of a HUSB reactor.

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1 Introduction

Microbial Fuel Cells (MFCs) 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). 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* (Richter et al., 2008; Kiely et al., 2011). 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 (Rabaey & Verstraete, 2005; Rabaey, et al., 2004; Nevin et al., 2008). 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 *Methanoseta* and *Methanosarcina* are, for example, routinely detected in mixed species, anode biofilms of MFCs, 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., 2014).

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, Rabaey and Verstraete, 2005). MFCs 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, MFCs 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 (García et al. 2003; Dusek et al. 2008; Pedescoll et al. 2013; Corbella et al., 2014). 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 (Yadav et al., 2012, Villaseñor et al., 2013; Fang et al., 2013; 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., 2001). 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.

2 Material and methods

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 m$^2$ 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.BOD₅.m⁻².day⁻¹ and 6 g.BOD₅.m⁻².day⁻¹ to the HUSB and Settler line, respectively.

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_MFCs) and the rest of them were
placed within the wetland fed by the settler (SET_MFCs) (Figure 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 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 that of 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 length, 3 cm wide and 1 cm height and square shaped (Figure
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.

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).

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 middle February to middle 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).

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-AWWA-WEF, 2005). Sampling was conducted at the inlet, middle and outlet of the wetlands on a weekly basis. Water flow was also daily measured and removal efficiencies calculated on a mass balance basis.

2.6 Electrochemical characterization

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

\[ P = \frac{V^2}{R} \]

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,

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

\[
V_{ef} = \frac{E_{cell}}{E_{emf}} \cdot 100
\]

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

2.7 Microbial community assessment

2.7.1 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.

2.7.2 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., in press). Standard curves were performed with known concentrations of the following reference cloned genes: 16S rRNA gene from *Desulfovibrio vulgaris* 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 -3.524 and -3.575 for 16S rRNA and mcrA gene, respectively.

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

2.7.3 454-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’-AGRRGTGTGATCMGGTGTCAG–3’) and 519R (5’-GNTTACNGCGCCGCTG-3’), while archaeal set of primers were 349F (5’-GYGCASCAGKCGMGAAW-3’) and 806R (5’–GGACTACVSGGGGTATCTAAT-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., 2010a) 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, Hugenholtz 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.

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. $E_{\text{cell}}$ was recorded by means of a datalogger (Datataker DT50 series 3) which collected a value of voltage across the external resistance every 15 minutes. $E_{\text{cell}}$ was measured for three days at each cathode to anode surface ratio tested.

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 below 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).

3 Results and discussion

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 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. 2013, Tanner 2001). Accordingly, water level variations impose higher redox conditions in wetlands which, in turn, may favour nitrification (García et al. 2003, García et al. 2010). In terms of organic matter, it was higher at the effluent of the HUSB reactor 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-MFCs which, in turn, could lead to a better performance of the cells (Cheng and Logan, 2011, Liu et al., 2004).

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 to 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.

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 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.

Concerning cell voltage, MFC replicates performed similarly for both lines (Figure 4).
Furthermore, it is widely accepted that cell electromotive force (\(E_{\text{emf}}\)) 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), showing picks during sunlight hours and being close to zero during nightly
hours. Although there was a notable variability on the picks, cell voltage started to rise
roughly between 11:00 and 15:00h and decreased between 18:00 and 23:00h.

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_MFCs achieved a maximum
cell voltage of 164 mV.

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 3) or cell voltage (Figure 4).
Similar patterns have been reported in current literature for MFCs implemented in
planted environments such as rice paddy fields (Kaku et al., 2008; De Schamphelaire et
al., 2008) or, more recently, in constructed wetlands (Villaseñor et al., 2013). So far, an
increase in the electrical output during sunlight hours is attributed to a higher
photosynthetic activity of plants that increases the total amount of substrate available (root exudates) for energy production (Strik et al., 2008; Kaku et al., 2008; De Schamphelaire et al., 2008). 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 in 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 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. 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).

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 6a and 6b). To this regard, from February until middle May, where temperature was that of 12.4 ± 4.4 °C 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 °C 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/m² and 25 mW/m² and 138 mA/m² and 14 mW/m² for the HUSB_MFCs and the SET_MFCs, 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/m² and 36 mW/m²). Average values for the first period were 82 ± 38 mA/m² and 6 ± 5 mW/m² and 66±37 mA/m² and 4 ± 4 mW/m² to the HUSB_MFCs and the SET_MFCs, respectively. Daily power production values (data not shown) followed also the same pattern than 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 ($E_{\text{cell}}$) compared to the maximum attainable ($E_{\text{emf}}$) was, in average, $13 \pm 12\%$ and $7 \pm 5\%$ to the HUSB and the settler line, respectively. Therefore, results suggested that MFC were highly limited.

Power and current densities described by Villaseñor et al. (2013) and Yadav et al. (2012) for MFC implemented in constructed wetlands are 43 mW/m$^2$ and of 16 mW/m$^2$ and 37 mA/m$^2$ and 70 mA/m$^2$, 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 7). From Figure 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.

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.

3.4.1 Total eubacteria and methanogenic populations abundance

Total microbial populations ranged from $2 \cdot 10^8$ to $6 \cdot 10^7$ 16S rRNA gene copies · 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 (See supplementary material Figure S1; Table 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 · 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.

### 3.4.2 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 (See supplementary material Table S2). However, the average cleaned reads taxonomically assigned as archaea per sample were from five to ten fold lower than those achieved for eubacteria (See supplementary material Table 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-Wiener (5.0-5.5), and even a higher evenness index (0.23-0.38) (See supplementary material Figure S2 and Table 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 8a and b (and also in Table S3). Settled wastewater showed a high predominance of beta-proteobacteria (average 29%) and Flavobacteria (average 48%) (Figure 8a; Supplementary material Table 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 (Fig. 8a and b; Table 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 (Fig. 8a and b; Table 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%).
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 (Fig. 8a; Table 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 8; Table S3 and 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 S5).

Regarding archaeal population, it is noteworthy the high relative prevalence of Methanosetaeaceae 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 Methanosetaeaceae 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 (Chrenarchaeota) 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 9a,b, and Figure S3-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
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-proteobacteria). 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 9 a,b and Fig S3-4), 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 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 *Methanoseta* has been just slightly enriched in the anode material of both experimental lines (Table S5). Current research is revealing the occurrence of exoelectrogenic activity in *Methanosarcina* and *Methanosaeta* sharing electrons with a concomitant *Geobacter*, (Rotaru et al., 2014 a, b) promoting potential electron current production in MFCs and complex microbial communities such as those harboured in natural environments.

4 Conclusions

The settler line slightly outperformed the HUSB line in terms of treatment efficiency, though only in terms of total COD differences were significantly different.
Maximum current and power densities recorded with microbial fuel cells implemented in constructed wetlands for the treatment of real domestic wastewater was that of 219 mA/m² and 36 mW/m².

Microbial fuel cells implemented in constructed wetlands receiving the effluent of an anaerobic reactor showed higher current and power densities than microbial fuel cells implemented in the wetlands receiving primary settled wastewater. However, differences among treatment lines were not significantly different. The lack of significant differences was probably due to a cathode surface limitation.

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 and exposed the cathode to air.

The type of primary treatment implemented had a significant impact on the diversity and relative abundance of bacteria 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.

5 Acknowledgements

This study was funded by the Spanish Ministry of Science and Innovation (MICINN) (project CTM2010-17750).

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Table 1. Physical and chemical parameters measured. *Note: average values are shown with standard deviation in brackets.*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HUSB</th>
<th>SETTLER</th>
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<tbody>
<tr>
<td></td>
<td>in</td>
<td>middle</td>
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<tr>
<td>DBO&lt;sub&gt;soluble&lt;/sub&gt; (n=7) (mg O&lt;sub&gt;2&lt;/sub&gt;/L)</td>
<td>137 (63)</td>
<td>71 (26)*</td>
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<tr>
<td>COD&lt;sub&gt;total&lt;/sub&gt; (n=17) (mg O&lt;sub&gt;2&lt;/sub&gt;/L)</td>
<td>323 (33)*</td>
<td>137 (53)*</td>
</tr>
<tr>
<td>AMMONIA (n=16) (mg NH&lt;sub&gt;4&lt;/sub&gt;^+ - N/L)</td>
<td>41 (7)</td>
<td>-</td>
</tr>
<tr>
<td>NITRATE (n=13) (mg NO&lt;sub&gt;3&lt;/sub&gt;-N/ L)</td>
<td>&lt; 1</td>
<td>-</td>
</tr>
<tr>
<td>NITRITE (n=13) (mg NO&lt;sub&gt;2&lt;/sub&gt;-N/ L)</td>
<td>&lt; 1</td>
<td>-</td>
</tr>
<tr>
<td>SULPHATE (n=13) (mg SO&lt;sub&gt;4&lt;/sub&gt;^2- /L)</td>
<td>102 (27)</td>
<td>-</td>
</tr>
<tr>
<td>ORTHOPHOSPHATE (n=13) (mg P-PO&lt;sub&gt;4&lt;/sub&gt;^3- /L)</td>
<td>9 (3)</td>
<td>-</td>
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* Significant differences among treatment lines.
Figure 1. Microbial fuel cells implemented within the wetland at the beginning of the experiment.

Figure 2. Outline of microbial fuel cells and electrodes.
Figure 3. Representative redox daily pattern for the HUSB line.

Figure 4. Representative voltage pattern recorded for microbial fuel cells implemented within the HUSB and the Settler line.
Figure 5. Representative cell voltage and water level variation for the HUSB line.
Figure 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 middle June since after then, only the HUSB line was left under operation.
Figure 7. Influence of cathode to anode surface ratio on $E_{\text{cell}}$. Note: error bars are the standard deviation of two replicates; each dot represents a three-day average value of the $E_{\text{cell}}$ recorded during sunlight hours.
Figure 8. Biodiversity of main representatives of eubacteria (sorted by class) (A) and archaea (sorted by family) (B) expressed as relative OTUs abundance (%). Thermoprotei class or Fervidococaceae 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 7B.
Figure 9: Covariance-based Principal Component Analysis biplot of a) eubacterial and b) archaeal OTUs distribution from pyrosequencing analysis from different samples.