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Title: 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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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

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

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18 Abstract

19 This work aimed at determining the amount of energy that can be harvested by
20 implementing microbial fuel cells (MFC) in horizontal subsurface constructed wetlands
21 (HSSF CWs) during the treatment of real domestic wastewater. To this aim, MFC were
22 implemented in a pilot plant based on two HSSF CW, one fed with primary settled
23 wastewater (Settler line) and the other fed with the effluent of a hydrolytic up-flow
24 sludge blanket reactor (HUSB line). The eubacterial and archaeal community was
25 profiled on wetland gravel, MFC electrodes and primary treated wastewater by means
26 of 16S rRNA gene-based 454-pyrosequencing and qPCR of *16S rRNA* and *mcrA* genes.
27 Maximum current (219 mA/m²) and power (36 mW/m²) densities were obtained for the
28 HUSB line. Power production pattern correlated well with water level fluctuations

29 within the wetlands, whereas the type of primary treatment implemented had a
30 significant impact on the diversity and relative abundance of eubacteria communities
31 colonizing MFC. It is worth noticing the high predominance (13-16% of relative
32 abundance) of one OTU belonging to *Geobacter* on active MFC of the HUSB line that
33 was absent for the settler line MFC. Hence, MFCs show promise for power production
34 in constructed wetlands receiving the effluent of a HUSB reactor.

35 Key words: constructed wetlands, domestic wastewater, microbial fuel cells, *Geobacter*
36

37 1 Introduction

38 Microbial Fuel Cells (MFCs) are bioelectrochemical systems that generate current by
39 means of electrochemically active microorganisms as catalysts. In a MFC, organic and
40 inorganic substrates are oxidized by bacteria and the electrons are transferred to the
41 anode from where they flow through a conductive material and a resistor to a higher
42 redox electron acceptor, such as oxygen, at the cathode (Logan et al., 2006, Rabaey et
43 al., 2007). So far, there are two well-known bacterial genera which present
44 exoelectrogenic activity in pure culture, i.e., *Shewanella* (Ringeisen et al., 2006) and
45 *Geobacter* (Richter et al., 2008; Kiely et al., 2011). To date, a high diversity of
46 microorganisms has been described to perform anode respiration to a certain degree
47 (Logan, 2009). Over 20 different exoelectrogenic bacteria have been reported in the last
48 decade, belonging to diverse phylogenetic groups: alpha-proteobacteria
49 (*Rhodopseudomonas*, *Ochrobactrum* and *Acidiphilium*), beta-proteobacteria
50 (*Rhodoferrax*, *Comamonas*), gamma-proteobacteria (*Shewanella*, *Pseudomonas*,
51 *Klebsiella*, *Enterobacter* and *Aeromonas*, *Citrobacter*), delta-proteobacteria
52 (*Geobacter*, *Geopsychrobacter*, *Desulfuromonas* and *Desulfobulbus*), Epsilon-
53 proteobacteria (*Arcobacter*), Firmicutes (*Clostridium* and *Thermincola*), Acidobacteria
54 (*Geothrix*) and Actinobacteria (*Propionibacterium*) (Logan 2009; Xing et al., 2010).
55 However, the power density achieved in most of the experiments working with mixed
56 cultures is higher than in pure cultures (Rabaey & Verstraete, 2005; Rabaey, et al.,
57 2004; Nevin et al., 2008). These results reinforce the idea that increased electricity
58 generation could be attributed to synergistic interactions within the microbial
59 community. Namely, there could be microorganisms that do not exchange directly
60 electrons with the electrode, but could be settling up interactions among other members

61 of the microbial community playing a crucial role not only in the operation of a MFC
62 but also on its performance improvement (specially under the presence of complex
63 organic substrates such as wastewater) (Borole et al., 2011 and references therein).
64 Methanogens such as *Methanosaeta* and *Methanosarcina* are, for example, routinely
65 detected in mixed species, anode biofilms of MFCs, where they presumably promote
66 syntrophic interactions with exoelectrogenic eubacteria in the anode biofilm (Chung and
67 Okabe, 2009; Rotaru et al., 2014a, 2014b; Sotres et al., 2014).

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

78 To generate the redox gradient between electrodes, MFCs require two separated areas
79 that contain the anode (anaerobic area) and the cathode (aerobic area). In some aquatic
80 environments there is the existence of natural redox gradients that can be exploited to
81 produce energy via MFC implementation. So far, MFC have been mostly implemented
82 in rice paddy fields (De Schamphelaire et al., 2008, Kaku et al., 2008) or marine
83 sediments (Reimers et al., 2001; Rezaei et al., 2007). Furthermore, horizontal
84 subsurface flow constructed wetlands (HSSF CWs) are engineered systems used for
85 wastewater treatment that are subjected to great spatial redox variations (especially in
86 depth) (García et al. 2003). Although the system is mainly anaerobic (Baptista et al.
87 2003), the very upper part of the wetland remains under aerobic conditions because its
88 close contact with the atmosphere giving redox gradients of about 0.5 V (García et al.
89 2003; Dusek et al. 2008; Pedescoll et al. 2013; Corbella et al., 2014). As a result, natural
90 redox gradients in HSSF CWs could be exploited to produce energy via MFC
91 implementation, though only laboratory or small-scale based experiments with synthetic
92 wastewater are currently available (Yadav et al., 2012, Villaseñor et al., 2013; Fang et
93 al., 2013; Zhao et al., 2013). Furthermore, one of the main problems of constructed
94 wetlands is clogging (Pedescoll et al. 2011a). To prevent it, primary treatments are

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

111 2 Material and methods

112 2.1 *Pilot plant*

113 The pilot plant used in this study consisted of two horizontal subsurface flow
114 constructed wetland. The wetlands were set up in March 2011 and had 0.4 m² of surface
115 (70 cm length x 55 cm width). Wetlands were filled up with gravel (D₆₀=7.3; C_u=0.8)
116 giving an initial porosity of 40%. Water level within the wetlands was kept at 30 cm (5
117 cm below the gravel surface). Both wetlands were planted with common reed
118 (*Phragmites australis*), which were very mature at the moment this study was
119 conducted (2.5 years after wetlands construction). Each wetland had a PVC cylinder of
120 20 cm diameter placed at the middle of the wetland that served not only to sample
121 extraction but also to allocate the MFC.

122 The pilot plant was fed with urban wastewater pumped directly from the municipal
123 sewer. Initially, wastewater was coarsely screened and after that it was pumped to a
124 homogenisation tank of 1.2 m³ where wastewater was continuously stirred in order to
125 avoid solids sedimentation. After the homogenisation tank, wastewater was conveyed to
126 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 copper 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 BOD₅, 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:

$$P = V^2/R$$

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 cm²) in order to express power production per wetland surface.

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

187
$$E_{emf} = E_{cat} - E_{an}$$

188 Where,

189 E_{cell} : cell voltage (in volts)

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

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

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

196
$$V_{eff} = \frac{E_{cell}}{E_{emf}} \cdot 100$$

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

199 2.7 Microbial community assessment

200 2.7.1 DNA extraction

201 Wastewater samples from settler and HUSB were filtered in triplicate (5 mL each
202 replicate) by means of Swinnex® Filter Holders (Millipore) with membrane filters of
203 cellulose acetate (Whatman® 0.22 µm pore diameter). Filtrates were kept frozen at -
204 20°C until DNA extraction. Total DNA was extracted from influent wastewater
205 filtrates, graphite material and gravel samples from both settler and HUSB lines. A bead
206 beating DNA extraction was performed in triplicate by means of PowerSoil® DNA
207 Isolation Kit (MoBio Laboratories, Solano Beach, CA, USA), following
208 manufacturer's instructions.

209

210 2.7.2 Quantitative PCR assay

211 The ratios between eubacterial and methanogenic archaeal population were determined
212 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'-AGRGTTCGATCMTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3'), while archaeal set of primers were 349F (5'-GYGCASCAGKCGMGAAG-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., 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).

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

247 2.8 *Assessment of cathode limiting conditions*

248 Results from the main experiment suggested that MFC performance was limited by the
249 cathode surface applied. In order to confirm this hypothesis a short experiment was
250 conducted at the end of the study period. The experiment consisted of increasing the
251 surface of cathode up to five times than that of the anode for two of the MFC
252 implemented in the HUSB line. More precisely, the cathode to anode surface ratios
253 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
254 datalogger (Datataker DT50 series 3) which collected a value of voltage across the
255 external resistance every 15 minutes. E_{cell} was measured for three days at each cathode
256 to anode surface ratio tested.

257 2.9 *Statistical analyses*

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

268

269 3 Results and discussion

270 3.1 *Treatment performance*

271 No differences were found between experimental lines for ammonia, nitrate, nitrite,
272 sulphate and orthophosphate. Ammonia removal was 60%, regardless the experimental
273 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, Ligeró et al. 2001). Moreover, samples extracted from the central part of the wetlands indicated that BOD₅, 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 °C, with minimum values in February (6 °C) and maximum values in July (28 °C). Moreover, water temperature followed a daily cycle with temperature variations of about 2 °C 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

307 differences among treatment lines. Redox potential at the cathode described a very
308 constant day-night pattern, especially pronounced for the HUSB line (Figure 3).
309 Accordingly, during nightly hours the redox potential at 5 cm depth was almost as
310 reduced as that of 15 cm depth and, therefore, the redox gradient was almost zero.
311 However, during sunlight hours the redox potential increased to a notable extent
312 reaching maximum values between 200 and 300 mV, regardless the experimental line
313 considered. Overall, and as has been previously described (Corbella et al., 2014), redox
314 gradient between the anode and the cathode ranged between 400 and 500 mV,
315 regardless the experimental line considered and, thus, no significant differences were
316 recorded among experimental conditions.

317 Concerning cell voltage, MFC replicates performed similarly for both lines (Figure 4).
318 Furthermore, it is widely accepted that cell electromotive force (E_{emf}) equals the
319 difference between the cathode and the anode potential (redox gradient) minus the
320 losses of the system (namely overpotentials and ohmic losses) (Logan et al., 2006). As
321 expected, voltage recorded followed the same daily pattern than the redox gradient
322 (Figure 4), showing picks during sunlight hours and being close to zero during nightly
323 hours. Although there was a notable variability on the picks, cell voltage started to rise
324 roughly between 11:00 and 15:00h and decreased between 18:00 and 23:00h.

325

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

332 3.3 *Effect of evapotranspiration on daily and seasonal cell performance*

333 As it has been pointed out in the previous section, daily oscillations were observed all
334 through the study period either in terms of redox (Figure 3) or cell voltage (Figure 4).
335 Similar patterns have been reported in current literature for MFCs implemented in
336 planted environments such as rice paddy fields (Kaku et al., 2008; De Schamphelaire et
337 al., 2008) or, more recently, in constructed wetlands (Villaseñor et al., 2013). So far, an
338 increase in the electrical output during sunlight hours is attributed to a higher

339 photosynthetic activity of plants that increases the total amount of substrate available
340 (root exudates) for energy production (Strik et al., 2008; Kaku et al., 2008; De
341 Schampelaire et al., 2008). However, water losses caused by evapotranspiration in
342 planted systems have been also described to influence MFC performance (Strik et al.,
343 2008). Although water level inside the wetlands was set to be 30 cm, significant water
344 level variations from the design value were observed all along the study period. More
345 precisely, water level within the wetlands decreased from the design level in 3.1 ± 0.9
346 cm (in May where plants started to grow) to 6.1 ± 1.8 cm (in July where plants were
347 already grown) (Figure 5). Moreover, intense water level variation, especially from May
348 until the end of the study period, left the cathode of the MFC exposed to the atmosphere
349 during the central hours of the day. When cathode was air-exposed, oxygen availability
350 increased and favoured the current generation due to an increase of the cell voltage. Our
351 results are in accordance with current literature since it is generally accepted that MFC
352 performance is related to oxygen availability at the cathode (Fan et al., 2008; Oh et al.,
353 2004).

354

355 Furthermore, our results suggested not only that cell voltage was influenced by water
356 level variation on a daily basis, but also in terms of seasonal variations (Figure 6a and
357 6b). To this regard, from February until middle May, where temperature was that of
358 12.4 ± 4.4 °C and plants were not yet developed, no significant power and current were
359 recorded. From middle May until the end of July, when temperature rose up to $21.1 \pm$
360 5.1 °C and plants were already developed, both current and power density started to
361 increase, reaching maximum values during the first period (both HUSB and settler lines
362 under operation) of 181 mA/m^2 and 25 mW/m^2 and 138 mA/m^2 and 14 mW/m^2 for the
363 HUSB_MFCs and the SET_MFCs, respectively. During the second period (only the
364 HUSB line) microbial fuel cells achieved the maximum power and current densities
365 recorded for the whole study period (219 mA/m^2 and 36 mW/m^2). Average values for
366 the first period were $82 \pm 38 \text{ mA/m}^2$ and $6 \pm 5 \text{ mW/m}^2$ and $66 \pm 37 \text{ mA/m}^2$ and 4 ± 4
367 mW/m^2 to the HUSB_MFCs and the SET_MFCs, respectively. Daily power production
368 values (data not shown) followed also the same pattern than the current and power
369 density, reaching maximum values during the first period of about $259 \text{ mWh.m}^{-2}.\text{day}^{-1}$
370 and $158 \text{ mWh.m}^{-2}.\text{day}^{-1}$ for the HUSB line and the settler line, respectively.

371

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

375

376 Power and current densities described by Villaseñor et al. (2013) and Yadav et al.
377 (2012) for MFC implemented in constructed wetlands are 43 mW/m^2 and of 16 mW/m^2
378 and 37 mA/m^2 and 70 mA/m^2 , respectively. Therefore, our results are in the range of
379 that previously reported in literature, though this is the first time that, to the knowledge
380 of the authors, MFC are implemented in pilot-scale wetlands treating real domestic
381 wastewater, where the availability of easy biodegradable substrates is of lesser extent
382 when compared to synthetic wastewater. Overall, despite the HUSB line showed higher
383 maximum power production when compared to the settler line, no significant
384 differences were recorded among treatment lines. It is important to point out that
385 authors believe that one of the reasons behind the lack of significant differences among
386 treatment lines concerning the average cell voltage recorded was the high oxygen
387 limitation at the cathode. Indeed, the experiment on the assessment of cathode limitation
388 conditions performed at the end of the study period confirmed that MFC operated
389 during the whole period of study were probably subjected to a cathode limitation
390 surface (Figure 7). From Figure 7 it is clear that in order to avoid any cathode limiting
391 condition the surface of cathode shall be around four times higher than that of the
392 anode.

393 *3.4 Effect of primary treatment on bacterial populations in MFC-implemented CW*

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

399 *3.4.1 Total eubacteria and methanogenic populations abundance*

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

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

435 encountered for the HUSB line samples when compared to the settler line based on
436 certain diversity estimators such as OTU numbers (640-775), Chao-1 (837-987),
437 Shannon-Wiener (5.0-5.5), and even a higher evenness index (0.23-0.38) (See
438 supplementary material Figure S2 and Table S2).

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

447 Biodiversity of eubacteria (by class) and archaea (by family) in terms of relative
448 incidence for the main taxonomic groups are shown in Figure 8a and b (and also in
449 Table S3). Settled wastewater showed a high predominance of beta-proteobacteria
450 (average 29%) and Flavobacteria (average 48%) (Figure 8a; Supplementary material
451 Table S3). Anaerobically pre-treated wastewater showed a predominance of beta and
452 gamma-proteobacteria (average 46% and 32%, respectively) and, to a lesser extent,
453 flavobacteria (6%) and clostridia (7%).

454 Samples analyzed for eubacteria from the wetland fed with the settler effluent
455 (including gravel and graphite at open and close circuit MFC) presented no significant
456 differences concerning the dominance of groups at class level (Fig. 8a and b; Table S3).
457 Among eubacteria dominant classes were that of alfa-proteobacteria (around 20-32%)
458 and Flavobacteria (around 16-30%).

459 Samples analyzed for eubacteria from within the wetland fed with the HUSB effluent
460 (including gravel and graphite at open and close circuit MFC) also showed no
461 significant differences concerning the dominance of groups at class level (Fig. 8a and b;
462 Table S3). Among eubacteria dominant classes were that of Alphaproteobacteria (up to
463 17%), Deltaproteobacteria (up to 30%); clostridia (up to 18%); bacterioidia (up to ca.
464 8%); synergistia (up to 9%) and anaerolineae (up to 6%).

465 Diversity of eubacteria was significantly higher, encompassing important phylogenetic
466 and quantitative changes, when the HUSB line was compared to the Settler line,
467 regardless the type of sample considered (Fig. 8a; Table S3). More precisely, for gravel
468 and graphite samples of the HUSB line predominant groups were that of clostridia,
469 delta-proteobacteria, Bacteroidia, Synergistia, alfa-proteobacteria and Anaerolineae
470 (Figure 8; Table S3 and S4). Accordingly, the fundamental difference in microbial
471 community structure promoted by the two types of primary treatment here considered
472 was the high enrichment in Bacteroidia (OTUs 1 and 2) and delta-proteobacteria class in
473 the gravel and graphite samples of the HUSB line (OTU 4) when compared to gravel
474 and graphite samples of the settler line.

475 Regarding delta-proteobacteria class it is remarkable the relative predominance of the
476 Geobacteraceae family in gravel and graphite samples of the HUSB line (19% and 5%
477 of relative abundance for the graphite of active MFC and gravel samples, respectively).
478 Within the Geobacteraceae family it is of special interest the high relative abundance of
479 one OTU belonging to *Geobacter* in active MFC of the HUSB line (from 13 to 16%). In
480 the case of gravel and graphite samples from the settler line, Geobacteraceae were not
481 only less favored (below 2%), but even the detected Geobacteraceae OTUs were
482 different from that of the OTUs found in samples from the HUSB line (Table S5).

483 Regarding archaeal population, it is noteworthy the high relative prevalence of
484 Methanosaetaceae family at the effluent of both types of primary treatments (55% and
485 81% for the settler and HUSB reactor, respectively). Furthermore, there was a shift in
486 methanogenic archaea that consisted in a high decrease of Methanosaetaceae
487 encompassed by an enrichment of Methanomicrobiaceae/Thermoplasmata (OTU2) as it
488 is assigned by Greengenes/RDP Bayesian Classifier in HUSB line. In addition, a non
489 methanogenic phylum (Chrenarchaeota) was highly predominant in gravel and
490 graphite samples from both experimental lines. (Chrenarchaeota, assigned as
491 Fervidicoccaceae/Thermoprotei by Greengenes/RDP Bayesian Classifier) (Wang et al.,
492 2007) (37-53% and 23-39% to the settler and HUSB lines, respectively).

493 Multivariate statistical analyses were conducted by means of covariate-principal
494 component analyses (PCA) (Figure 9a,b, and Figure S3-S4) and correspondence
495 analyses (CA) with similar results. PCA and CA analysis revealed the existence of three
496 main separate groups of samples encompassing different microbial communities

(primary treated wastewater samples, settler line and HUSB line) (Figure 9 a,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-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. Coincidentally, 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 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.

528 Maximum current and power densities recorded with microbial fuel cells implemented
529 in constructed wetlands for the treatment of real domestic wastewater was that of 219
530 mA/m² and 36 mW/m².

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

536 Redox gradient between electrodes and cell voltage followed a very conservative
537 pattern along the day with higher output cell voltage values during daylight hours. The
538 main parameter controlling the cell voltage was water level variation within the
539 wetlands that resulted from intense evapotranspiration and exposed the cathode to air.

540 The type of primary treatment implemented had a significant impact on the diversity
541 and relative abundance of bacteria communities colonizing MFC. It is worth noticing
542 the high predominance (13-16% of relative abundance) of one OTU belonging to
543 *Geobacter* on active MFC of the HUSB line that was absent for the settler line MFC.

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547 6 References

548 Álvarez, J. a., Ruíz, I., Soto, M, 2008. Anaerobic digesters as a pretreatment for
549 constructed wetlands. Ecological Engineering 33(1), 54–67.

550 APHA-AWWA-WEF, 2005. Standard Methods for the Examination of Water and
551 Wastewater. 21st ed. American Public Health Association, Washington, DC.

552 Arroyo, P., Ansola, G., Miera. L.E., Effects of substrate, vegetation and flow on arsenic
553 and zinc removal efficiency and microbial diversity in constructed wetlands.
554 Ecological Engineering 51, 95-103.

555 Baptista, J. D. C., Donnelly, T., Rayne, D., Davenport, R. J., 2003. Microbial
556 mechanisms of carbon removal in subsurface flow wetlands. *Water Science and*
557 *Technology* □: A Journal of the International Association on Water Pollution
558 Research 48(5), 127–34.

559 Borole, A. P.; Reguera, G.; Ringeisen, B.; Wang, Z.-W.; Feng, Y.; Kim, B. H., 2011.
560 Electroactive biofilms: Current status and future research needs. *Energy Environ.*
561 *Sci.* 4, 4813-4834

562 Calheiros, C. S., Duque, A. F., Moura, A., Henriques, I. S., Correia, A., Rangel, A. O.,
563 Castro, P. M., 2009. Changes in the bacterial community structure in two-stage
564 constructed wetlands with different plants for industrial wastewater treatment.
565 *Bioresource technology*, 100(13), 3228-3235.

566 Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K.
567 Costello, N. Fierer, A. Gonzalez-Pena, J.K. Goodrich, J.I. Gordon, G.A. Huttley,
568 S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald,
569 B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A.
570 Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, R. Knight, 2010a. QIIME
571 allows analysis of high-throughput community sequencing data. *Nature Methods*
572 7, 335-336.

573 Caporaso, J.G., K. Bittinger, F.D. Bushman, T.Z. DeSantis, G.L. Andersen, R. Knight,
574 2010b. PyNAST: A flexible tool for aligning sequences to a template alignment.
575 *Bioinformatics* 26, 266-267.

576 Cheng, S., Logan, B. E., 2011. Increasing power generation for scaling up single-
577 chamber air cathode microbial fuel cells. *Bioresource Technology*, 102(6), 4468–
578 73.

579 Chung K and Okabe S, 2009. Continuous power generation and microbial community
580 structure of the anode biofilms in a three-stage microbial fuel cell system. *Applied*
581 *Microbiology and Biotechnology* 83: 965-977.

582 Clauwaert, P., Aelterman, P., Pham, T. H., De Schampelaire, L., Carballa, M., Rabaey,
583 K., Verstraete, W., 2008. Minimizing losses in bio-electrochemical systems: the
584 road to applications. *Applied Microbiology and Biotechnology* 79(6), 901–13.

585 Corbella, C., Garfí, M., Puigagut, J., 2014. Vertical redox profiles in treatment wetlands
586 as function of hydraulic regime and macrophytes presence: Surveying the optimal
587 scenario for microbial fuel cell implementation. *Science of the Total*
588 *Environment*, 470-471, 754–8.

589 DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., ...
590 Andersen, G. L., 2006. Greengenes, a chimera-checked 16S rRNA gene database
591 and workbench compatible with ARB. *Applied and Environmental Microbiology*,
592 72(7), 5069–72.

593 De Schamphelaire, L., Van den Bossche, L., Dang, H. S., Höfte, M., Boon, N., Rabaey,
594 K., Verstraete, W., 2008. Microbial fuel cells generating electricity from
595 rhizodeposits of rice plants. *Environmental Science & Technology*, 42(8), 3053–
596 8.

597 Dong, X., Reddy, G. B., 2010. Soil bacterial communities in constructed wetlands
598 treated with swine wastewater using PCR-DGGE technique. *Bioresource*
599 *technology*, 101(4), 1175-1182.

600 Dušek, J., Pícek, T., Čížková, H., 2008. Redox potential dynamics in a horizontal
601 subsurface flow constructed wetland for wastewater treatment: Diel, seasonal and
602 spatial fluctuations. *Ecological Engineering*, 34(3), 223–232.

603 Du, Z., Li, H., Gu, T., 2007. A state of the art review on microbial fuel cells: A
604 promising technology for wastewater treatment and bioenergy. *Biotechnology*
605 *Advances*, 25(5), 464–82.

606 Fang, Z., Song, H.-L., Cang, N., Li, X.-N. 2013. Performance of microbial fuel cell
607 coupled constructed wetland system for decolorization of azo dye and
608 bioelectricity generation. *Bioresource Technology*, 144, 165–71.

609 Fan, Y., Sharbrough, E., & Liu, H., 2008. Quantification of the internal resistance
610 distribution of microbial fuel cells. *Environmental Science & Technology*, 42(21),
611 8101–7.

612 García, J., Ojeda, E., Sales, E., Chico, F., Píriz, T., Aguirre, P., Mujeriego, R., 2003.
613 Spatial variations of temperature, redox potential, and contaminants in horizontal
614 flow reed beds. *Ecological Engineering*, 21(2-3), 129–142.

615 García, J., Rousseau, D. P. L., Morató, J., Lesage, E., Matamoros, V., Bayona, J. M.,
616 2010. Contaminant Removal Processes in Subsurface-Flow Constructed
617 Wetlands: A Review. *Critical Reviews in Environmental Science and*
618 *Technology*, 40(7), 561–661.

619 Gonçalves, R. F., Charlier, A. C., Sammut, F., 1994. Primary fermentation of soluble
620 and particulate organic matter for wastewater treatment. *Water Science and*
621 *Technology: A Journal of the International Association on Water Pollution*
622 *Research*, 30(6), 53–62.

623 Imfeld, G., Aragonés, C. E., Fetzer, I., Mészáros, É., Zeiger, S., Nijenhuis, I., ...
624 Richnow, H. H., 2010. Characterization of microbial communities in the aqueous
625 phase of a constructed model wetland treating 1, 2-dichloroethene-contaminated
626 groundwater. *FEMS microbiology ecology*, 72(1), 74-88.

627 Kaku, N., Yonezawa, N., Kodama, Y., Watanabe, K., 2008. Plant/microbe cooperation
628 for electricity generation in a rice paddy field. *Applied Microbiology and*
629 *Biotechnology*, 79(1), 43–9.

630 Kiely P.D, Regan J.M, Logan B.E., 2011. The electric picnic: synergistic requirements
631 for exoelectrogenic microbial communities. *Current Opinion in Biotechnology*,
632 22:378-385.

633 Lefebvre, O., Uzabiaga, A., Chang, I. S., Kim, B.-H., Ng, H. Y., 2011. Microbial fuel
634 cells for energy self-sufficient domestic wastewater treatment-a review and
635 discussion from energetic consideration. *Applied Microbiology and*
636 *Biotechnology*, 89(2), 259–70.

637 Ligeró, P., Vega, A., Soto, M., 2001. Pretreatment of urban wastewaters in a hydrolytic
638 upflow digester, 27(3), 399–404.

639 Liu, H., Ramnarayanan, R., Logan, B. E., 2004. Production of electricity during
640 wastewater treatment using a single chamber microbial fuel cell. *Environmental*
641 *Science & Technology*, 38(7), 2281–5.

642 Lladó, S., Covino, S., Solanas, A.M., Petruccioli, M., D’annibale, A. and Viñas M.,
643 2015. Pyrosequencing reveals the effect of mobilizing agents and lignocellulosic

644 substrate amendment on microbial community composition in a real industrial
645 PAH-polluted soil. *Journal of Hazardous Materials*, 283, 35-43

646 Logan, B. E., Hamelers, B., Rozendal, R., Schröder, U., Keller, J., Freguia, S., Rabaey,
647 K., 2006. Microbial fuel cells: methodology and technology. *Environmental*
648 *Science & Technology*, 40(17), 5181–92.

649 Logan, B.E., 2009. Exoelectrogenic bacteria that power microbial fuel cells. *Nature*
650 *Reviews in Microbiology*, 7, 375-381.

651 Min, B., Logan, B. E., 2004. Continuous electricity generation from domestic
652 wastewater and organic substrates in a flat plate microbial fuel cell.
653 *Environmental Science & Technology*, 38(21), 5809–14.

654 Nevin KP, Richter H, Covalla SF, Johnson JP, Woodard TL, Orloff AL, Jia H, Zhang M
655 and Lovley DR., 2008. Power output and coulombic efficiencies from biofilms of
656 *Geobacter sulfurreducens* comparable to mixed community microbial fuel cells.
657 *Environ Microbiol* 10: 2505-2514

658 Oh, S., Min, B., Logan, B. E., 2004. Cathode performance as a factor in electricity
659 generation in microbial fuel cells. *Environmental Science & Technology*, 38(18),
660 4900–4.

661 Pedescoll, A., Corzo, A., Alvarez, E., García, J., Puigagut, J., 2011a. The effect of
662 primary treatment and flow regime on clogging development in horizontal
663 subsurface flow constructed wetlands: An experimental evaluation. *Water*
664 *Research*, 45(12), 3579–89.

665 Pedescoll, A., Corzo, A., Álvarez, E., Puigagut, J., García, J., 2011b. Contaminant
666 removal efficiency depending on primary treatment and operational strategy in
667 horizontal subsurface flow treatment wetlands. *Ecological Engineering*, 37(2),
668 372–380.

669 Pedescoll, A., Sidrach-Cardona, R., Sánchez, J. C., Bécares, E., 2013.
670 Evapotranspiration affecting redox conditions in horizontal constructed wetlands
671 under Mediterranean climate: Influence of plant species. *Ecological Engineering*,
672 58, 335–343.

673 Rabaey K, Boon N, Siciliano S.D, Verhaege M and Verstraete W., 2004. Biofuel cells
674 select for microbial consortia that self-mediate electron transfer. *Applied and*
675 *Environmental Microbiology* 70:5373–5382.

676 Rabaey, K., & Verstraete, W., 2005. Microbial fuel cells: novel biotechnology for
677 energy generation. *Trends in Biotechnology*, 23(6), 291–8.

678 Rabaey, K., Rodríguez, J., Blackall, L. L., Keller, J., Gross, P., Batstone, D., Nealon,
679 K. H., 2007. Microbial ecology meets electrochemistry: electricity-driven and
680 driving communities. *The ISME Journal*, 1(1), 9–18.

681 Reimers, C. E., Tender, L. M., Fertig, S., Wang, W., 2001. Harvesting energy from the
682 marine sediment--water interface. *Environmental Science & Technology*, 35(1),
683 192–5.

684 Rezaei, F., Richard, T. L., Brennan, R. a, Logan, B. E., 2007. Substrate-enhanced
685 microbial fuel cells for improved remote power generation from sediment-based
686 systems. *Environmental Science & Technology*, 41(11), 4053–8.

687 Richter H, Mc Carthy K, Nevin KP, Johnson JP, Rotello VM and Lovley DR, 2008.
688 Electricity generation by *Geobacter sulfurreducens* attached to gold electrodes.
689 *Langmuir* 24: 4376-4379.

690 Ringeisen BR, Henderson E, Wu PK, Pietron J, Ray R, Little B, Biffinger JC and Jones-
691 Meehan JM, 2006. High power density from a miniature microbial fuel cell using
692 *Shewanella oneidensis* DSP10. *Environmental Science & Technology* 40: 2629-
693 2634.

694 Roche Diagnostics. 2009. Using multiple identifier (MID) adaptors for the GS FLX
695 titanium chemistry-extended MID set. Technical Bulletin: Genome Sequencer
696 FLX System.

697 Rotaru A, Shrestha P, Liu F, Shrestha M, Shrestha D, Embree M, Zengler K, Wardman
698 C, Nevin KP and Lovley DR, 2014a. A new model for electron flow during
699 anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the
700 reduction of carbon dioxide to methane. *Energy Environ Sci* 7: 408-415.

701 Rotaru, A. E., Shrestha, P. M., Liu, F., Markovaite, B., Chen, S., Nevin, K., Lovley, D.,
702 2014b. Direct interspecies electron transfer between *Geobacter metallireducens*
703 and *Methanosarcina barkeri*. *Applied and environmental microbiology*, AEM-
704 00895.

705 Sotres, A, Diaz-Marcos, J., Guivernau, M., Illa, J., Magrí, A., Prenfeta-Boldú, F.X.,
706 Bonmatí, A., Viñas, M., 2014. Microbial community dynamics in two-chambered
707 microbial fuel cells: effect of different ion exchange membranes'. *Journal of*
708 *Chemical Technology and Biotechnology*. In Press DOI 10.1002/jctb.4465.

709 Strik, D. P. B. T. B., Snel, J. F. H., Buisman, C. J. N., 2008. SHORT
710 COMMUNICATION Green electricity production with living plants and bacteria
711 in a fuel cell, (January), 870–876.

712 Tanner, C. C., 2001. Plants as ecosystem engineers in subsurface-flow treatment
713 wetlands. *Water Science and Technology*: A Journal of the International
714 Association on Water Pollution Research, 44(11-12), 9–17.

715 Villaseñor, J., Capilla, P., Rodrigo, M. a, Cañizares, P., & Fernández, F. J., 2013.
716 Operation of a horizontal subsurface flow constructed wetland--microbial fuel cell
717 treating wastewater under different organic loading rates. *Water Research*, 47(17),
718 6731–8.

719 Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for
720 rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl.*
721 *Environ. Microbiol.* 73, 5261–5267.

722 Xing, D., Cheng, S., Logan, B.E., Regan, J.M., 2010. Isolation of the exoelectrogenic
723 denitrifying bacterium *Comamonas denitrificans* based on dilution to extinction.
724 *Applied Microbiology and Biotechnology*, 85, 1575-1587

725 Yadav, A. K., Dash, P., Mohanty, A., Abbassi, R., Mishra, B. K., 2012. Performance
726 assessment of innovative constructed wetland-microbial fuel cell for electricity
727 production and dye removal. *Ecological Engineering*, 47, 126–131.

728 Zhao, Y., Collum, S., Phelan, M., Goodbody, T., Doherty, L., Hu, Y., 2013. Preliminary
729 investigation of constructed wetland incorporating microbial fuel cell: Batch and
730 continuous flow trials. *Chemical Engineering Journal*, 229, 364–370.

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

		HUSB				SETTLER			
		in	middle	out	% removal	in	middle	out	% removal
DBO _{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 NH ₄ ⁺ -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 E (n=13)	(mg P-PO ₄ ³⁻ /L)	9 (3)	-	10 (7)	-	7 (2)	-	5 (5)	-

* Significant differences among treatment lines.

1

2 Figure 1. Microbial fuel cells implemented within the wetland at the beginning of the
3 experiment.

4

5 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.

27

28 Figure 6. Daily maximum current (a) and power (b) density evolution along the
 29 experiment for the HUSB_MFCs and the SET_MFCs. Note that values from the settler
 30 line are only plotted until middle June since after then, only the HUSB line was left
 31 under operation.

Figure 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.

A)

B)

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 Feravidicocacceae 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.