SUSTAINABLE POWER PRODUCTION IN A MEMBRANE-LESS AND MEDIATOR-LESS WASTEWATER MICROBIAL FUEL CELL

Aba Aldrovandi [a], Enrico Marsili [b], Loredana Stante [a], Patrizia Paganin [c], Silvia Tabacchioni [c], Andrea Giordano [a,*]

[a] ENEA C.R. Bologna, ACS PROTIDR Section, Via Martiri di Montesole 4, 40129 Bologna – Italy

[b] BioTechnology Institute, University of Minnesota, 1479 Gortner Ave, St. Paul, Minnesota 55108 - USA

[c] ENEA C.R. Casaccia, BAS-BIOTEC-GEN Section, Via Anguillarese 301, S. Maria di Galeria 00123 Roma - Italy

* Corresponding author. Tel.: +39-051-6098580; Fax: +39-051-6098309

Email address: andrea.giordano@bologna.enea.it (A. Giordano)

Abstract

Microbial fuel cells (MFCs) fed with wastewater are currently considered a feasible strategy for production of renewable electricity at low cost.

A membrane-less MFC with biological cathode was built from a compact wastewater treatment reactor. When operated with an external resistance of 250 Ohm, the MFC produced a long-term power of approximately 70 mW/m^2 for ten months. Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the cathode biomass when the MFC was closed on a 2100 Ohm external resistance showed that the sequenced bands were affiliated with *Firmicutes*, α-*Proteobacteria,* β-*Proteobacteria*, γ-*Proteobacteria,* and *Bacteroidetes* groups.

When the external resistance was varied between 250 and 2100 Ohm, sustainable resistance decreased from 900 to 750 Ohm, while sustainable power output decreased from 32 to 28 mW/m^2. It is likely that these effects were caused by changes in the microbial ecology of anodic and cathodic biomass attached to the electrodes. Results suggest that cathodic biomass enrichment in "electroactive" bacteria may improve MFCs power output in a similar fashion to what has been already observed for anodic biomass.

Keywords: microbial fuel cell, sustainable power, biocathode, PCR-DGGE.

1. Introduction

In a Microbial Fuel Cell (MFC), microorganisms convert chemical energy to electrical energy, via microbial-catalyzed redox reactions. A typical MFC consists of anode and cathode compartments separated by a cationic membrane. Microbes in the

anode compartment oxidize a soluble electron donor (e.g., glucose, acetate) generating electrons and protons. Electrons are then transferred to the anode surface and from there to the cathodic compartment through the electrical circuit, while the protons migrate through the electrolyte and then through the cationic membrane. Electrons and protons are consumed in the cathode compartment reducing a soluble electron acceptor, such as oxygen or ferricyanide. Electrical power is harnessed by inserting a load between the two electrode compartments (Allen and Bennetto, 1993).

In early studies, exogenous electrochemicals mediators have been added into the MFCs to allow electron transfer from the microbial cells to the electrode, due to the non-conductive nature of the cell surface structures (Roller et al., 1984; Park and Zeikus, 2003; Logan and Regan, 2006). However, recent evidence (Kim et al., 2004; Jang et al., 2004; Gil et al., 2003) showed that complex microbial communities in wastewater-fed MFCs produce soluble redox mediators (e.g., pyocyanin; Rabaey et al., 2004). Furthermore, *Geobacter sulfurreducens* is known to transfer electrons beyond cell surfaces to electrodes through membrane proteins (Bond and Lovley, 2003; Chaudhury and Lovley, 2003) or nanowires (Reguera et al., 2005), *Shewanella oneidensis* MR1 was shown to produce both soluble redox mediators (Marsili et al., 2008) and nanowires (Gorby et al., 2006).

It has been shown that wastewaters of different origin can be fed to MFCs, thus allowing energy production from an abundant and inexpensive source. Most of the energy available from the oxidation of the organic load was converted to electricity (50÷90 % in term of Coulombic efficiency) while the remaining energy was used for microbial growth (Liu et al., 2007; Rabaey et al., 2005a). Integration of MFCs in wastewater treatment plants allowed energy recovery and reduction of excess sludge

production with little effect on the mineralization of organic load and the rest of the process (Rabaey and Verstraete, 2005). Wastewater MFCs are currently being assessed as a renewable energy strategy (Logan and Regan, 2006).

However, in order to make the process economically feasible, it is necessary to: a) eliminate the cationic membrane to reduce operating costs due to membrane maintenance; b) implement MFCs in existing wastewater treatment plants, to reduce capital investment; c) avoid an expensive cathodic catalyst in favour of aerobic biomass (He and Angenent, 2006).

Recently, membrane-less wastewater MFCs have been designed and tested (Jang et al., 2004; Moon et al., 2006; Ghangrekar and Shinde, 2007). While many studies have dealt with anodic compartment (Min and Logan, 2004; You et al., 2006; Liu et al., 2004), a few studies have been performed both to develop a biocathode capable of directly reducing oxygen and to characterize the microbial community responsible for the cathode catalysis (Bergel et al., 2005; Chen et al., 2008; Clauwaert et al., 2007; Kang et al., 2003, Rabaey et al., 2008).

In this study, a membrane-less, mediator-less MFC was implemented on a compact lab-scale wastewater treatment plant (WWTP), with a simple and economic design, to couple wastewater treatment (removal of organic compounds and ammonia oxidation) with sustainable electrical power production to avoid the use of expensive catalysts for the cathode. The effect of the external load on sustainable power production was investigated. Furthermore, the microbial composition of cathodic biomass was characterized in order to determine whether the long-term operation of the MFC cause an enrichment in electroactive bacteria at the cathode. The results indicate

that the electroactivity of cathodic biomass affect the power production in completely biological MFCs.

2. Material and Methods

2.1 Process and reactor design

The two-stage process shown in Fig. 1 was adopted for organic substrate removal and ammonia nitrification of high strength wastewaters (Malina and Pohland, 1992; Tchobanoglous et al., 2003). Sedimentation and fermentation of easily degradable organic substrate took place in the first anaerobic stage of the process, and the following aerobic stage nitrified ammonia and further oxidized organic compounds.

The bench-scale WWTP was built in PVC, composed of two anaerobic compartments arranged in series (ABR, Anaerobic Baffled Reactor), and an aerobic chamber and a sludge settler (implementing an activated sludge process), as shown in Fig. 1. The volumes of the four compartments were 18 L, 18 L, 22 L, and 5 L, respectively. The anaerobic compartments #1 and #2 were connected through an overfall. A recycle pump (Watson Marlow 503 S, Wilmington MA, USA) was placed between the two anaerobic compartments to evenly distribute the liquid below the sludge blanket. Constant temperature in the ABR (35°C) was maintained with a heating element (Rena, France) located in compartment #1 and controlled by a temperature probe (Tersid, Milano, Italy) in compartment #2. The return activated sludge was recycled from the settler #4 to the oxidation chamber #3 (Recycle ratio R_r=2). The aerobic compartment #3 was aerated and stirred with an aquarium air pump (Schego, Germany), with an air flow rate of approximately 6 L/min.

2.2 Inoculation and medium

Anaerobic compartments #1 and #2 were inoculated with 15.2 L of granular sludge from a full-scale Up-flow Anaerobic Sludge Blanket (UASB) reactor treating paper factory wastewater (Castelfranco Emilia, Italy). The aerobic compartment #3 was inoculated with 6 L of activated sludge from a full-scale domestic wastewater treatment plant (Trebbo di Reno, Italy).

The plant was fed with synthetic wastewater with the following composition (per liter of tap water): 168 mg $(NH_4)_2SO_4$, 60 mg $MgSO_4 \cdot 7H_2O$, 6 mg $MnSO_4 \cdot H_2O$, 126 mg $NaHCO_3$, 0.3 mg $FeCl_3 \cdot 6H_2O$, and 6 mg $CaCl_2 \cdot 2H_2O$. Anhydrous glucose was used as the organic substrate, at different concentrations as shown in Table 1. The synthetic wastewater was buffered at pH 7.3 with 0.7 M K_2HPO_4 and 0.3 M KH_2PO_4. All chemicals were of reagent grade. Synthetic wastewater was stored and fed to the reactor at 4°C and replaced every 3-4 days.

2.3 Chemical analyses

A pH electrode (Mettler Toledo, OH, USA) was placed in the anaerobic compartment #2. Biogas production rate from the two anaerobic compartments was monitored through a custom prepared wet tip gas flow meter. Methane content in biogas was analyzed with an Ultramat 23 infrared spectrophotometer (Siemens, NY, USA). Oxidation-reduction potential (ORP) was measured in both anaerobic (#2) and aerobic compartments (#3) with metallic electrodes (Crison model 52-61, Alella, Spain). Dissolved oxygen (DO) concentration in compartment #3 was measured with a Cell OX 325 oxymetric sensor (WTW OXI 340, Weilheim, Germany). Chemical Oxygen Demand (COD) and ammonia nitrogen were determined in samples collected from compartment #2 and #4, according to the Standard Methods (APHA, 1999).

2.4 Microbial fuel cell

Untreated glassy carbon anode and cathode, with geometrical surface of 160 cm^2 each, were cleaned overnight in 1 M HCl and rinsed with deionized water prior to use, then placed in compartment #2 and #3, respectively (Fig. 1). Electrodes were connected via an external electric circuit on a load variable between 150 and 2000 Ohm. A saturated calomel electrode (SCE, Hanna Instruments, RI, USA), was placed in compartment #3 and used as reference to measure anodic and cathodic electrochemical potentials.

Cell and half cell potentials were measured every hour with a milli-voltmeter (Datataker, UK). Current was calculated from the potential through a shunt resistance of 100 Ohm. Temperature, pH, redox potentials, biogas production and electric measures were stored in a Datataker 605 (Datataker, UK). The total applied resistance is reffered as the sum of external variable load and shunt resistance (100 Ohm), since the shunt was in series with the load on the external circuit.

Sustainable power measure was performed daily with an in-house built programmable variable resistance, as described by Menicucci et al., (2006). In a typical experiment, total applied resistance varied between 1800 and 110 Ohm, with a rate of 400 Ohm/min. This procedure returned the maximum sustainable power (MSP), which is the highest electrical power produced at steady state conditions. In addition to maximum sustainable power, the minimum sustainable external resistance and the maximum sustainable cell potential were determined.

2.5 MFC Operating conditions

Following inoculation, the reactor was continuously fed for 14 months. Changes in operating conditions during this time period are shown in Table 1. The synthetic wastewater was fed to the anaerobic compartment #1 of the MFC, with an organic load

(OL) variable between 0 and 16 g_{COD}/day, maintaining an anaerobic hydraulic residence time of 12 days. The anode was never changed during the experiment, while a clean cathode was inserted on day 238.

2.6 Microbiological analyses

2.6.1 Sample collection and DNA extraction

During step #8 suspended activated sludge (about 50 ml) was collected from the aerobic chamber and sludge attached on the cathode was removed using a sterile razor blade and re-suspended in a sterile phosphate buffer saline solution. Samples were stored at - 80°C until use.

Sludge samples (5 ml) were dispersed overnight with a cation-exchange resin (Chelex 100, Biorad, CA, USA) as described by Frølund et al. (1996). Lysis of the cells was performed by the lysozyme + SDS technique described by Bourrain et al., (1999). Total DNA from microbial cells was obtained by phenol/chloroform extraction and isopropanol precipitation methods as previously described (Zhou et al., 1996). Purification of crude DNA extracts was performed by the Wizard DNA clean-up system (Promega, WI, USA). DNA concentration was determined spectrophotometrically with NanoDrop instrument ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA).

2.6.2 DGGE analysis of the V3 region of the 16S rRNA gene

Two µl (10 to 30ng of DNA) of each sample were subjected to PCR amplification of the 16S rRNA gene using Bacteria-specific primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTTGTTACG A-3') as described by Di Cello et al., (1997) to produce a 1.450-bp fragment, which was then used as a template for nested PCR with two different sets of primers. The first set, composed of primers 357F-GC-clamp (5'-

CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGG CAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3'), amplified a fragment of 161 bp (small fragment). The second set, consisted of primer 63F-GC-clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCAGGCCTAAC ACATGCAAGTC-3') and the reverse primer 518R described above, was used to produce a 495 bp fragment (large fragment). Nested PCRs to obtain the small and the large fragments were carried out according to the procedures described by Van der Gucht et al., (2005) and by El Fantroussi et al., (1999), respectively, with Taq DNA polymerase (Quiagen, Milano, Italy). Fragments were resolved by double gradient denaturing gradient gel electrophoresis (DG-DGGE) as described by Cremonesi et al., (1997), in a DCode universal mutation detection system (Bio-Rad, CA, USA). A 6 %-12 % polyacrylamide (acrylamide: N,N-methylenebisacrylamide, 37.5:1) gel with denaturing gradient ranging from 30 % to 60 % was used to resolve small fragments, whereas a 6 %-12 % polyacrylamide (acrylamide: N,N-methylenebisacrylamide, 37.5:1) gel with denaturing gradient ranging from 40 % to 70 % was used to resolve large fragments. A 100 % denaturing solution contained 7 M urea and 40 % deionized formamide. Approximately 700 ng of purified PCR products were loaded in each well. The gels were run for 16 h at 75 mV in 1X TAE buffer at 60°C, stained with 50 µg/ml ethidium bromide for 30 min, destained in water and photographed with the UVIpro Platinum Gel Documentation System (GAS7500/7510).

2.6.3 Sequencing of DGGE fragments and phylogenetic analysis

DGGE-separated fragments were excised with a razor blade and allowed to passively diffuse into the water at 4°C overnight. The eluted DNA was reamplified with the same primers and PCR conditions described for DGGE analysis. The PCR products were

tested by DGGE for purity and identity with the original bands in the community profiles and then sequenced. Sequencing reactions were prepared using Applied Biosystem Big Dye® Terminator sequencing kit version 3.1, according to the manufacturer's instructions and analyzed using a 3730 DNA Analyzer Applied Biosystem apparatus. Each sequence was submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU) to detect the presence of possible chimeric artifacts. Sequence similarity searches were performed using the BLAST network service of the NCBI database and Seqmatch tool of the RDP (http://www.ncbi.nlm.nih.gov/BLAST/ and http://rdp.cme.msu.edu/, respectively). For phylogenetic analysis, identification of 16S rRNA gene sequences was performed with RDP Classification Algorithm (http://rdp.cme.msu.edu/classifier/classifier.jsp).

Partial 16S rRNA gene sequences obtained in this study have been deposited in the NCBI nucleotide database under accession numbers EU492873-76 (small fragment bands A-D) and EU597325-31 (large fragment bands F-N).

3. Results

3.1 COD and ammonia removal

The influent and effluent organic load during phase I to III (see Table 1) are shown in Fig. 2, while Table 2 shows the COD removal in anaerobic and aerobic stages of the process, as well as methane production in anaerobic stage. During phase I, the influent glucose concentration was increased from 1.5 to 5 g/L (steps #1 to #4), corresponding to a measured feeding OL of 3.4 to 16.0 g_{COD}/day. The average COD removal in the anaerobic compartment varied between 66 and 91 % (Table 2). The

lowest value of COD removal was observed during the initial adaptation of anaerobic biomass.

During phase II (step #5, see Table 2), glucose was removed from the influent and the reactor was operated in absence of carbon and energy source. During this phase, the COD values in the outlet were lower than 70 mg_{COD}/L.

During phase III (step #6 and #7), glucose was added again to the influent, at a concentration of 3 g/L, corresponding to a measured OL of approximately 9.1 g_{COD}/day. COD removal in anaerobic compartment rapidly stabilized at 94 % (Table 2). The anaerobic COD removal measured in step #3, step #6 and #7 increased, although the feeding OL was the same. This was due to the anaerobic biomass growth, consequent to the absence of sludge withdrawal during the experimental period.

Daily methane production (Table 2) increased with OL and the organic substrate removed in the ABR chamber (#1 and #2) was almost completely converted into methane. The average pH value in the second ABR chamber was 6.9.

Dissolved oxygen in the aerobic compartment was 6.5±0.3 mgO_2/L through all the experiment, while ammonia nitrogen in the effluent was always below than 1 mg NH_4-N/L, corresponding to 98 % nitrogen removal.

3.2 Electrical power production

Immediately after inoculation, the cell was closed on a total applied resistance of 250 Ohm for a period of 270 days. The cell potential grew from 50±3 mV at the end of the starting phase (step #1), to 411±10 mV at the end of step #3 (Table 3), corresponding to an OL of 9.6 g_{COD}/day (and a substrate concentration in the second anaerobic chamber of 420 g_{COD}/m^3, Table 2). Further OL increase from 9.6 to 16 g_{COD}/day (815 g_{COD}/m^3 in the second anaerobic chamber, Table 2 step #4) did not

change the cell potential. Current and power output showed a similar pattern, reaching a plateau of 163±5 mA/m^2 and 65.1±3.5 mW/m^2 of geometric electrode surface at substrate concentration higher than 420 g_{COD}/m^3, indicating that organic substrate concentration was not limiting power output.

When glucose was removed from medium for two weeks (step #5), power production dropped rapidly reaching 1.2±0.3 mW/m^2, indicating that electricity was produced mainly by the catalytic oxidation of organic substrate. During step #6, the reactor was operated with an OL of 9.5 g_{COD}/day and the electrical power production reached in 14 days a plateau value of 72.7± 1.6 mW/m^2. Specific current, cell potential specific power, anodic and cathodic potential throughout the experiment are shown in Fig. 3 a, Fig. 3 b, Fig. 3 c and Table 3.

3.3 Anodic and cathodic potential

At OL higher than 6.4 g_{COD}/day (step #2), anode and cathode potential reached a plateau of -176± 5 and +234± 5 mV vs. SCE, respectively (Fig. 3 c and Table 3). In the absence of organic substrate (step #5), anodic potential increased to +237± 28 mV vs. SCE, while cathode potential was less affected, indicating that the cathode was not the current-limiting electrode in the cell (i.e. the one of the two electrodes that exhibits the slower charge-transfer kinetics). Both specific power output and anode potential were restored at nearly 100 % of their maximum values upon reintroduction of substrate in the medium (step #6, Fig. 3 b and Fig. 3 c), suggesting that the anodic attached biomass maintained its substrate oxidation capability.

3.4 Role of cathodic biomass

Cathodic potential dropped rapidly to -214± 1.4 mV vs. SCE when a clean glassy carbon cathode was inserted in the aerobic compartment (step #7). However,

residual electrical power was 10.5±0.1 mW/m^2, suggesting that suspended biomass and soluble redox mediators had a role in the cathodic process (Fig. 3 c and Fig. 3 b). Although thermodynamically feasible, direct oxygen reduction at the glassy carbon electrode is not possible at the measured potential, as it was also shown during the acclimation of cathodic biomass (step #1) The clean cathode was quickly colonized by aerobic biomass, and after 5 days cell potential and power production resumed to 100 ± 10% and 100 ± 3.3% of their values measured at the end of step #4, respectively (Table 3 and Fig. 3 b).

To investigate the composition of the microbial community suspended in the aerobic chamber and attached on the cathode, a partial domains profile of eubacterial 16S rRNA gene was performed by DGGE. This was done after PCR amplification of the 16S rDNA genes from total DNA of the two samples with the two sets of primers described in Materials and Methods. Fig. 4 a and Fig. 4 b shows the DGGE patterns of the small and large 16S rDNA fragments. There are fewer bands in the DGGE profiles of the small fragment than in the DGGE patterns of the large fragment. However, suspended aerobic biomass and the cathode attached biomass showed high fingerprint similarity as most of the bands obtained were present in both samples (Fig. 4 a and Fig. 4 b). Separated DGGE bands were excised from the gels, purified to determine the sequence, and assigned to a specific group on the basis of a combination of Blast searches and phylogenetic analysis. Table 4 shows the percentage of similarity between sequences of excised bands and the closest relatives (NCBI Database). The nucleotide sequences of the small fragment bands were referred to uncultured bacteria or DGGE clones of the *Firmicutes*, *β-Proteobacteria*, and *Bacteroidetes* groups (Table 5), whereas most of the sequences of the large fragment bands were affiliated with α-*Proteobacteria* and γ-

Proteobacteria groups. A band (E) was identified as a putative chimeric artefact based on result from CHECK_CHIMERA analyses. Moreover, two large fragment bands (G and H) were distantly affiliated with unclassified bacterial sequences retrieved from a river sediment sample (EF667579), from a commercial nitrifying inoculum (AJ786605.1) and from a biocathode chamber in a microbial fuel cell (EU 426928.1), (Table 4).

3.5 Sustainable resistance measure

Sustainable resistance was measured at four different values of total applied resistance, 250, 430, 1100, and 2100 Ohm, for at least 30 days. When the total applied resistance was changed, the average value of sustainable resistance changed as shown in Fig. 5 a. Specifically, at low total applied resistance (250 Ohm), the sustainable resistance decrease rapidly with time, while at high total applied resistance (2100 Ohm), the sustainable resistance value did not change appreciably with time (data not shown). Sustainable power slightly increased when total resistance decreased (Fig. 5 a).

3.6 Factor limiting power production

3.6.1 Current-limiting electrode

The sustainable resistance was determined through an external resistance scan. Anode and cathode potentials were measured at each external resistance value. The electrode whose potential changes more during the scan is the current-limiting electrode (Menicucci et al., 2006; Fuel cells handbook, 2004). Fig. 5 b and Fig. 5 c show a typical external resistance scan between 110 and 1800 Ohm. The cathodic potential was nearly constant at each external resistance value, showing that the current production was limited by the anode.

3.6.2 Limiting step in electron transfer

Current did not show a maximum during the resistance scan, while power reached a maximum at low value of external resistance (Fig. 5 c). A recent model proved that these trends are typical of a MFC in which diffusion processes limits power output (Shimotori et al., 2007). Because of these limitations, the sustainable power is less than half the maximum power (Fig. 5 a). Diffusion limitations to electron transfer are predominant in the range of total applied resistance explored in this study, which is between 250 and 2100 Ohms.

4. Discussion

4.1 Power production

The cell was initially operated with a total applied resistance of 250 Ohm. After 58 days of biomass growth and with non-limiting OL, cathode and anode potential reached a stable plateau of 234±5 mV and -176±5 mV vs. SCE, respectively (Fig. 3 c).

Both anodic and cathodic biomass established slowly and a plateau in current and power production was reached only after 70 days (i.e. after about 3.3 times the total Hydraulic Retention Time, HRT). Anodic attached biomass maintained its catalytic activity over two weeks of feeding without glucose (step #5). When a new cathode was inserted in the cell (step #7) suspended biomass adhered to the electrode in 5 days fully restoring the catalytic reducing activity and the half cell potentials.

The potential of the biocathode, about 230 mV vs. SCE (474 mV vs. SHE), was closed to that (~ 463 mV vs. SHE) observed for oxygen abiotic reduction at conventional Pt-coated electrodes in cathode chamber (Oh et al., 2004). Therefore, biocathode proved to be an efficient catalyst for oxygen reduction, and a feasible alternative to abiotic systems in wastewater-fed MFCs.

Concerns arose in the past over non-catalytic power production in wastewater fed MFC. Several media used for bacterial growth contained significant amount of redox mediators, such as cysteine, and high strength wastewater contained reduced sulfur species, which can work as abiotic electron donor and increase power production in short term experiments. In this study, minimal salt media was used, therefore electron donors other than glucose or redox mediators could be only by products of glucose anaerobic degradation or microbially produced electron shuttles (Rabaey et al., 2004, pyocyanine). In fact, when glucose was removed from the artificial wastewater fed to the MFC (step#5), the power dropped, indicating a discharge to the anode of the electrons stored in the attached biomass (e.g., in the protein membranes) and the reoxidation of most of the reduced species in solution.

The MFC was initially run under demanding conditions for current production (low external resistance). During this phase, glucose concentration in the wastewater and OL were increased until non-limiting conditions were achieved. Since further experiments were run using higher external resistance, it is assumed that non-limiting conditions with respect to glucose concentration were maintained. With a non limiting organic substrate concentration of 434±77 g_{COD}/m^3 in the second ABR chamber, the specific current increased in an exponential fashion and then stabilized at 159 ± 1 mA/m^2, while power reached a plateau at 60.6 ± 1 mW/m^2 (Fig. 3 a and Fig. 3 b).

Organic substrate removed in the ABR was converted almost completely into methane, as shown in Table 2. This results was expected due to the low ratio of electrode surface to the volume of anodic compartment ($S_{electrode}/V_{anode\ compartment}$ = 0.4 m^2/m^3). In other studies, different electrode material

configurations were adopted in order to maximize power output such as, granular graphite (Rabaey et al., 2005b), or high specific surface electrodes (Logan et al., 2006; Gil et al., 2003; Chaudhury and Lovley, 2003). In such systems (Rabaey et al., 2005c), an average COD to current efficiency of 59± 4 % was obtained and no methane production was observed.

4.2 Electron transfer mechanism

When attached cathodic biomass was removed (step #7), power production dropped to 13 % of the maximum power formerly measured. As discussed above, this observation suggested that microbially produced redox mediators facilitated electron transfer from the suspended and attached biomass to the anode. Microbially produced redox mediators were identified in at leas two cases (Rabaey et al., 2004; Marsili et al., 2008). It is not clear from our results whether the increase in cathodic potential and power production following electrode colonization is due to the concentration of such microbial redox mediators in the attached biomass, or if direct electron transfer modes are possible, as shown for dissimilatory metal-reducing bacteria (Reguera et al., 2005; Gorby et al., 2006). However, the residual power measured seems to indicate the co-presence of mediated and direct electron transfer mechanisms.

4.3 Factors limiting power production

The current-limiting electrode was determined through the sustainable power measures. In a typical external resistance scan, between 1800 and 110 Ohm, cathodic potential decrease by 20 % while the anodic potential increase by 70 % (Fig. 5 b). Once the cathodic biomass had developed, the anode was the current-limiting electrode. Sustainable power determination also provided insight over the processes which limit long-term power. When an MFC is limited by diffusion, the current measured during a

sustainable resistance scan always increases as external resistance decreases, although with a rate lower than that calculated from Ohm's law, since the cell potential is not constant (Menicucci et al., 2006). However, in MFCs with membrane electrode assembly (MEA) (Liang et al., 2007), power production is less affected by diffusion, and is rather limited by the amount of the immobilized biomass and its catalytic activity (Marsili et al., 2008; Shimotori et al., 2007). Sustainable power increases as long-term external resistance decreases, and sustainable resistance decreases as long-term external resistance decreases. Since decreased external resistance corresponds to higher electron flow at the attached biomass/electrode interfaces, it is likely that such conditions favor species with faster electron transfer rate. At higher external resistance, electroactive bacteria are no longer favored and biomass nor efficient in substrate utilization is likely selected.

DGGE of cathodic biomass using two different set of primers provided an analytical tool to study the diversity of the microbial community suspended and attached on the cathode. Use of the primers set P357F-GC clamp and 518R revealed a high fingerprint similarity between the two samples. However, it is often not easy to assign short partial sequences accurately, especially if the sequences lack close relatives in the database (Kuske et al., 1997). Because of this reason and to overcome PCR bias that often has been reported in DGGE experiments, (Hansen et al., 1998), we used the second set of primer (p63F-GC clamp-518R). The DGGE profiles obtained by this different set, that generated a larger 495-bp fragment, again revealed that the genomic fingerprintings of the two samples are very similar. Moreover, the use of both primer sets allowed a more accurate discrimination of different taxonomic groups inside the samples. In related studies that examined the microbial community present at the

biocathode, a similar breadth of phylogenetic diversity was detected (α, β, γ *Protobacteria*, *Bacteroidetes*) (Clauwert et al., 2007; Chen et al., 2008; Rabaey et al., 2008), whereas members of the *Firmicutes* group were detected for the first time. The presence of this bacterial taxon in the cathodic compartment could be related to the fact that the system was fed with glucose.

Conclusions

Further studies are needed to better understand the effect of external resistance on microbial composition. It is possible that increased power production corresponds to increased organization in electron transfer network in attached biomass. Also shift in electron transfer strategies (e.g., from mediated to direct electron transfer) may play a role in the change of sustainable resistance and sustainable power with time.

Based on the results from this study, we can conclude that with simple modifications, a common wastewater treatment plant can produce electrical power, without changes in COD removal and ammonia nitrification. The power produced was limited by diffusion phenomena, rather than from catalytic activity of electrode biomass. Sustainable power measures indicated that at lower external resistance electroactive bacteria are favored.

Biocathode is proved to be an efficient catalyst for oxygen reduction, and a feasible alternative to expensive Pt-based catalysts in wastewater-fed MFCs. A taxonomic characterization performed on both suspended and attacched cathodic biomass showed that the sequenced DGGE bands were affiliated with *Firmicutes*, α-*Proteobacteria,* β-*Proteobacteria*, γ-*Proteobacteria,* and *Bacteroidetes* groups.

References

Allen, R.M., Bennetto, H.P. (1993). Microbial fuel-cells: electricity production from carbohydrates. Appl. Biochem. Biotech., 39, 27–40.

APHA (1999). Standard Methods for the Examination of Water and Wastewater, 19th ed. Amer. Public Health Assoc., Washington, D.C. (1995).

Bergel, A., Fèron, D., Mollica, A (2005). Catalysis of oxygen reductionin PEM fuel cell by seawater biofilm. Elettrochemistry. Communications 7, 900-904.

Bond, D. R., Lovley, D. R. (2003). Electricity Production by *Geobacter Sulfurreducens* Attached to Electrodes. Appl. Environ. Microbiol., 69 (3), 1548-1555.

Bourrain, M., Achouak, W., Urbain, V., Heulin, T. (1999). DNA Extraction from Activated Sludges. Current Microbiol. 38 (6), 315-319.

Chaudhuri, S. K., Lovley, D. R. (2003). Electricity Generation by Direct Oxidation of Glucose in Mediatorless Microbial Fuel Cells. Nat. Biotechnol., 21 (10), 1229-1232.

Chen, G.W., Choi, S.J., Lee, T.H., Lee,G.Y.,Cha, J.H., Kim, C.W. (2008). Application of biocathode in microbial fuel cells: cell performance and microbial community. Appl. Microbiol. Biotechnol. 79, 379-388.

Clauwaert, P., Van Der Ha, D., Boon, N., Verbeken, K.,Verhaege, M., Rabaey, K., Verstraete, W. (2007). Open air biocathode enables effective electricity generation with microbial fuel cells. Environ. Sci. Technol. 41, 7564-7569.

Cremonesi, L., Firpo, S., Ferrari, M., Righetti, PG., Gelfi, C. (1997). Double-gradient DGGE for optimized detection of DNA point mutations. BioTechniques, 22 (2), 326-330.

Di Cello, F., Bevivino, A., Chiarini, L., Fani, R., Paffetti, D., Tabacchioni, S., Dalmastri, C. (1997). Biodiversity of a *Burkholderia Cepacia* population isolated

from the maize rhizosphere at different plant growth stages. Appl. Environ. Microbiol. 63 (11), 4485-4493.

El Fantroussi, S., Verschuere, L., Verstraete, W., Top, E. M. (1999). Effect of Phenylurea Herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. Appl. Environ. Microbiol. 65 (3), 982-988.

Frølund B., Palmgren R., Keiding K., Nielsen PH. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Res. 30 (8), 1749–1758.

Fuel Cell Handbook (Seventh Edition) by EG&G Technical Services, Inc. for the National Energy Technology Lab (NETL). 2004.

Ghangrekar, M.M., Shinde, V.B. (2007). Performance of membrane-less microbial fuel cell treating wastewater and effect of electrode distance and area on electricity production. Bioresour. Technol., 98 (15), 2879-2885.

Gil, G.C., Chang, I.S., Kim, B.H., Kim, M., Jang, J.K., Park, H.S., Kim, H.J. (2003). Operational parameters affecting the performance of a mediator-less microbial fuel cell. Biosens. Bioelectron. 18 (4), 327–334.

Gorby, Y. A., Yanina, S., Mclean, J. S., Rosso, K. M., Moyles, D., Dohnalkova, A., Beveridge, T. J., Chang, I. S., Kim, B. H., Kim, K. S., Culley, D. E., Reed, S. B., Romine, M. F., Saffarini, D. A., Hill, E. A., Shi, L., Elias, D. A., Kennedy, D. W., Pinchuk, G., Watanabe, K., Ishii, S., Logan, B., Nealson, K. H., Fredrickson, J. K. (2006). Electrically Conductive Bacterial Nanowires Produced by *Shewanella Oneidensis* Strain Mr-1 and Other Microorganisms. Proc. Natl. Acad. Sci. U. S. A. 103 (30), 11358-11363.

Jang, J. K., Pham, T. H., Chang, I. S., Kang, K. H., Moon, H., Cho, K. S., Kim, B. H. (2004). Construction and Operation of a Novel Mediator-and Membrane-Less Microbial Fuel Cell. Process Biochem. 39 (8), 1007-1012.

Hansen, M. C., Nielsen T. T., Givskov, M., Molin, S. (1998). Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. FEMS Microbiol. Ecol. 26 (2), 141-149.

He, Z., Angenent, L. T. (2006). Application of Bacterial Biocathodes in Microbial Fuel Cells. Electroanal. 18 (19-20), 2009-2015.

Kang, K. H., Jang, J. K., Pham, T. H., Moon, H., Chang, I. S., Kim, B. H. (2003). A Microbial Fuel Cell With Improved Cathode Reaction as a Low Biochemical Oxygen Demand Sensor. Biotechnol. Lett. 25 (16), 1357-1361.

Kim, B.H., Park, H.S., Kim, H.J., Kim, G.T., Chang, I.S., Lee, J., Phung, N.T. (2004). Enrichment of Microbial Community Generating Electricity Using a Fuel-Cell-Type Electrochemical Cell. Appl. Microbiol. Biotechnol. 63 (6), 672-681.

Kuske, C. R., Barns, S. M., Busch, J. D. (1997). Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. Appl. Environ. Microbiol. 63 (9), 3614-3621.

Liang, P., Huang, X., Fan, M. Z., Cao, X. X., Wang, C. (2007). Composition and Distribution of Internal Resistance in Three Types of Microbial Fuel Cells. Appl. Microbiol. Biotechnol. 77 (3), 551-558.

Liu, H., Ramnarayanan, R., Logan, B. E. (2004). Production of Electricity During Wastewater Treatment Using a Single Chamber Microbial Fuel Cell. Environ. Sci. Technol., 38 (7), 2281-2285.

1 Liu, Z.D., Du, Z.W., Lian, J., Zhu, X.Y., Li, S.H., Li, H.R. (2007). Improving energy
2 accumulation of microbial fuel cells by metabolism regulation using *Rhodoferax*
3 *ferrireducens* as biocatalyst. Appl. Microbiol. Biotechnol., 44 (4), 393–398.
4 Logan, B.E., Hamelers, B., Rozendal, R., Schroder, U., Keller, J., Freguia, S.,
5 Aelterman, P., Verstraete, W., Rabaey, K. (2006). Microbial Fuel Cells:
6 Methodology and Technology. Environ. Sci. Technol. 40 (17), 5181-5192.
7 Logan, B.E., Regan, J.M. (2006). Electricity-producing Bacterial Communities in
8 Microbial Fuel Cells. Trends in Biotechnol. 14 (12), 512-518.
9 Malina, F.J., Pohland, F.G. (1992). Design of Anaerobic Process for the Treatment of
10 Industrial and Municipal Wastes. Water Quality Management Library, Vol. 7, 169.
11 Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., Bond, D.R.
12 (2008). *Shewanella* Secretes Flavins That Mediate Extracellular Electron Transfer.
13 Proceeding National Academy of Sciences. 105 (10), 3968–3973.
14 Menicucci, J., Beyenal, H., Marsili, E., Veluchamy, R.A., Demir, G., Lewandowski, Z.
15 (2006). Procedure for Determining Maximum Sustainable Power Generated by
16 Microbial Fuel Cells. Environ. Sci. Technol. 40 (3), 1062-1068.
17 Min, B., Logan, B. E. (2004). Continuous Electricity Generation From Domestic
18 Wastewater and Organic Substrates in a Flat Plate Microbial Fuel Cell. Environ. Sci.
19 Technol., 38 (21), 5809-5814.
20 Moon, H., Chang, I. S., Kim, B. H. (2006). Continuous Electricity Production From
21 Artificial Wastewater Using a Mediator-Less Microbial Fuel Cell. Bioresour.
22 Technol., 97 (4), 621-627.
23 Oh S., Min B., Logan B. (2004). Cathode performance as a factor in elecrticity
24 generation in microbial fuel cell. Environ. Sci. Technol., 38 (18), 4900-4904.

Park, D. H. Zeikus, J. G. (2003). Improved Fuel Cell and Electrode Designs for Producing Electricity From Microbial Degradation. Biotechnol. Bioeng. 81 (3), 348-355.

Rabaey, K., Boon, N., Siciliano, S.D., Verhaege, M., Verstraete, W. (2004). Biofuel Cells Select for Microbial Consortia that Self-Mediate Electron Transfer. Appl. Environ. Microbiol., 70 (9), 5373–5382.

Rabaey, K., Verstraete, W. (2005). Microbial Fuel Cells: Novel Biotechnology for Energy Generation. Trends Biotechnol. 23 (6), 291-298.

Rabaey, K., Ossieur, W., Verhaege, M., Verstraete, W. (2005a). Continuous Microbial Fuel Cells Convert Carbohydrates to Electricity. Water Sci. Technol. 52 (1-2), 515-523.

Rabaey, K., Boon, N., Hofte, M., Verstraete, W. (2005b). Microbial Phenazine Production Enhances Electron Transfer in Biofuel Cells. Environ. Sci. Technol. 39 (9), 3401-3408.

Rabaey, K.,Clauwaert, P., Aelterman, P., Verstraete, W. (2005c). Tubular Microbial Fuel Cells for Efficient Electricity Generation. Environ. Sci. Technol., 39 (20), 8077-8082.

Rabaey, K., Read, S. T , Clauwaert, P., Freguia, S., Bond, P. L., Blackall, L. L., Keller, J. (2008). Cathodic oxygen reduction catalyzed by bacteria in microbial fuel cells. ISME J. 2, 519-527.

Reguera, G., Mccarthy, K. D., Mehta, T., Nicoll, J. S., Tuominen, M. T., Lovley, D. R. (2005). Extracellular Electron Transfer Via Microbial Nanowires. Nature, 435 (7045), 1098-1101.

Roller, S. D., Bennetto, H. P., Delaney, G. M., Mason, J. R., Stirling, J. L., Thurston, C.F. (1984). Electron-Transfer Coupling in Microbial Fuel-Cells .1. Comparison of Redox-Mediator Reduction Rates and Respiratory Rates of Bacteria. J. Chem. Technol. Biotechnol. B-Biotechnology, 34 (1), 3-12.

Shimotori, T., Bond, D.R., Lapara, T.M., Hozalski, R.M. (2007). Model of microbial fuel cells in which bacteria employ a direct electron transfer strategy. 234th ACS Natl. Meet. Book Abstr., 19 August 2007 - 23 August 2007.

Tchobanoglous, G., Burton, F.L., Stensel, H.D. (2003). Wastewater Engineering: Treatment and Reuse. 4th ed. Metcalf & Eddy Inc., New York, NY: McGraw-Hill.

Van der Gucht, K., Vandekerckhove, T., Vloemans, N., Cousin, S., Muylaert, K., Sabbe, K., Gillis, M., Declerk, S., De Meester, L., Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. FEMS Microbiol. Ecol. 53 (2), 205-220.

You, S. J., Zhao, Q. L., Jiang, J. Q., Zhang, J. N. (2006). Treatment of Domestic Wastewater With Simultaneous Electricity Generation in Microbial Fuel Cell Under Continuous Operation. Chem. Biochem. Eng. Q. 20 (4), 407-412.

Zhou, J., Bruns, M.A., Tiedgje, J.M. (1996). DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62 (2), 316-322.

Acknowledgment

The Authors would like to thank Prof. Natuschka M. Lee at the Technical University of Munich, Germany, for her discussion on microbial characterization.