Supplementary Material

Title: Design and optimization of Microbial Fuel Cell and evaluation of a new air-breathing cathode based on carbon felt modified with a hydrogel - Ion Jelly®

1) **DNA Extraction, sequencing, and sequence data analysis**

DNA extraction, sequencing, and data analysis of the microbial consortium composition of the samples collected during this work were performed by DNA Sense (Aalborg, Denmark).

Table S1. The microbial composition of the samples collected from the wastewater treatment plant streams (Chelas, Lisbon city, Portugal) and the biofilm on the carbon felt surface of MFC anodic reactors (R1 and R2).

Sample ID	Description
1	W1 control sample
2	W2 control sample
3	Wastewater treatment
4	Wastewater treatment
5	R1 Anodic chamber
6	R1 Anodic chamber
7	R1 Anodic chamber
8	R1 Anodic chamber
9	R2 Anodic chamber at the beginning of MFC function
10	R2 Anodic chamber

DNA extraction and sequencing

Total DNA was extracted from samples using the kit Fast DNA™ SPIN for Soil (MP Biomedicals, USA). The quality of the extracted DNA was evaluated after agarose gel electrophoresis, using the Tapestation 2200 and Genomic DNA screentapes (Agilent, USA). DNA concentration was estimated using the Qubit™ dsDNA BR Assay (Thermo Fisher Scientific, USA).

Bacterial 16S rRNA amplicon sequencing targeting the V1-3 variable regions was performed based on the methods of Caporaso et al. (2012) [39], using primers adapted from the Human Gut Consortium [40]. For this purpose, 10 ng of extracted DNA was used as the template. PCR reactions were carried out in a final volume of 25 µL, containing 400 nM dNTPs, 1.5 mM $MgSO_4$, 2 mU Platinum® Taq DNA polymerase, 1 X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA), and 400 nM barcoded library adaptors containing the V1-3 specific primers 27F (AGAGTTTGATCCTGGCTCAG) and 534R (ATTACCGCGGCTGCTGG). PCR amplification conditions were: initial denaturation at 95°C for 2 min; 30 cycles of 95°C for 20 s followed by 56°C for 30 s and 72°C for 60 s; and a final elongation step at 72°C for 5 min. All PCR reactions were run in duplicate. After amplification, the reaction mixtures were pooled, and the amplicon libraries were purified using the Agencourt® AMpure XP bead protocol from Beckmann Coulter (USA). Library concentration was measured with Quant-iT™ HS DNA Assay (Thermo Fisher Scientific, USA), and their quality was validated with a Tapestation 2200, using D1K ScreenTapes (Agilent, USA).

The samples, pooled in equimolar concentrations and diluted to 4 nM, were pair-end sequenced (2x301bp) on a MiSeq (Illumina, USA) device using a MiSeq Reagent kit v3 (Illumina, USA), according to standard guidelines for sample preparation and loading.

Sequence data analysis

Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 [41] with the settings SLIDINGWINDOW:5:3 and MINLEN:275 and merged using FLASH v. 1.2.7 [42] with the settings -m 25 -M 200. Merged reads were dereplicated and formatted for use in the UPARSE workflow [43], and clustered using the usearch v. 7.0.1090 -cluster_otus command with default settings. The command usearch v. 7.0.1090 -usearch_global with -id 0.97 was used to estimate the abundance of operational taxonomic units (OTUs). The RDP classifier [44] was used to assign taxonomy to the OTUs, as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME [45], using the MiDAS database v.1.23 [46]. The results were analyzed in R (R Core Team, 2017) [47] through the Rstudio IDE using the ampvis package v.2.2.8 [48].

2) **MFC abiotic cathode and biocathode configurations**

Operational stability and performance

The MFCs with abiotic cathode and biocathode configurations operated over approximately 230 and 195 days, respectively, corresponding to about 10 cycles of synthetic effluent feeding and three additional biomass inoculum injections (Fig S1).

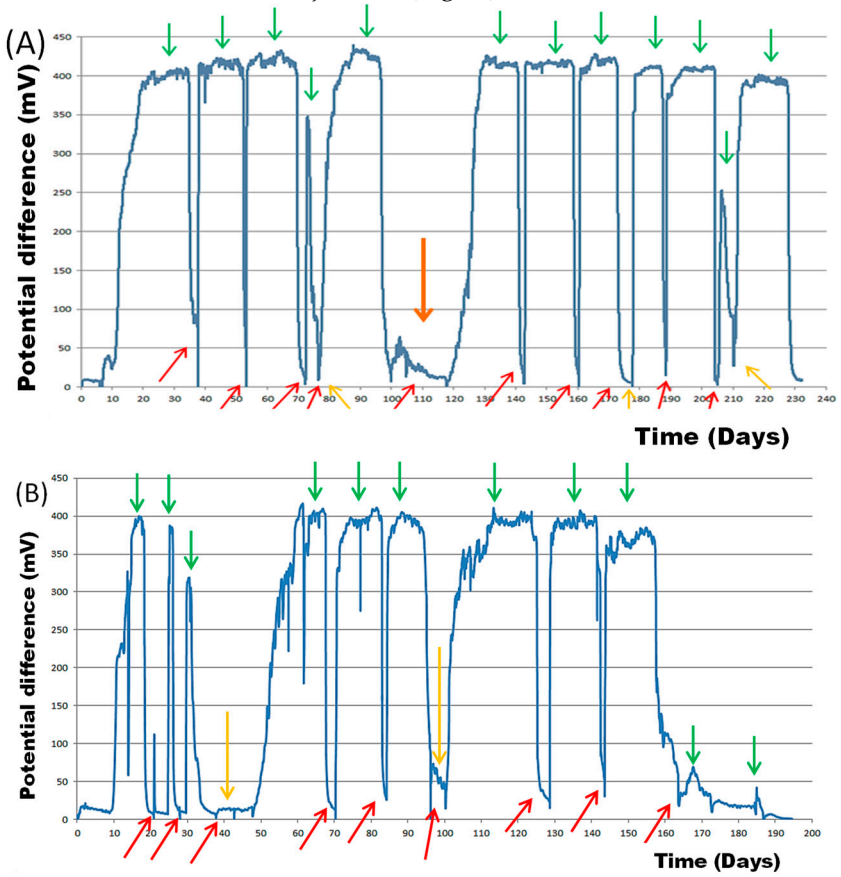

Figure S1. The potential difference over time. (A) MFC with abiotic cathode configuration. (B) for MFC biocathode configuration. The green and red arrows represent the beginning and end of each cycle, the yellow arrows correspond to biomass inoculum injection, and the orange arrow identifies an academic pause.

The end of the electric generation of both MFC configurations seems to be associated with the total saturation and covered of the anode electrode surface with a compact brown biofilm. This biofilm creates high resistance to mass transfer and is impenetrable to colonize with fresh

bacteria. Reusing the carbon felt after a washing process that can remove the biofilm formed is an interesting issue to be checked. The extended stability and high performance of both MFC configurations confirm the authors' previous work [36].

A new biomass inoculum injection into the anodic compartment was performed when the feed of synthetic effluent was not enough to recover the electrical energy production. However, after new biomass inoculation, the microorganisms proliferate again in the anodic compartment, refreshing the biomass film on the electrode surface and quickly starting a new electrical energy generation cycle (Fig. S1).

The break of energy generation at day 100 (orange arrow) was due to the academic pause, which did not allow us to proceed with the regular renewal of the synthetic effluent for at least 10 days (Fig. S1). Despite the carbon source absence and even after conditions of great metabolic stress, the electric current production restarted with a new feed of synthetic effluent in the anodic compartment on day 112. The electric generation proceeded over three more cycles between days 120 and 200 (Fig. S1).

Both MFC configurations presented a promising electricity production performance using the microbial consortium collected from a wastewater treatment station. The bioelectricity production cycles were stable on an average of 10 to 15 days, and the larger cycle reached 20 days (Fig. S1).

The MFC abiotic cathode configuration achieved a maximal voltage of 440 mV, with DP reaching a maximum of 54 mW/m^2, DC of 122 mA/m^2, and OCV of 561 mV. The MFC biocathode configuration led to a maximum voltage of 417 mV, a DP of 48 mW/m^2, a DC of 116 mA/m^2, and an OCV of 534 mV.

The samples ID 7 and 8 in Table S1 were collected from the biofilms of the anode electrodes surface of the MFC abiotic cathode and biocathode configurations, respectively, after 4/5 cycles, i.e., about 80 hours of electric generation.

3) **COD reduction efficiencies**

The COD reduction and Coulombic Efficiency (CE) for both MFC configurations were assayed and calculated. The wastewater treatment efficiencies evaluated by COD reduction were 91% and 93%, and CE of 31% and 34% for the MFCs abiotic cathode and biocathode, respectively. These results confirmed that both MFCs had a similar wastewater treatment efficiency, but the biocathode proved slightly more efficient than the abiotic cathode, also observed by other authors [36,63]. The COD removal efficiency was greater than 90% for both MFC configurations, with results comparable to other studies [36,60,63]. The CE for both MFC configurations was around 30%, which is reasonable considering that similar values were obtained in many other studies [36,60].

If the objective of the MFC performance is the degradation of organic matter (measured by COD reduction) by microorganisms consortium immobilized on the carbon felt electrode surface, the biocathode should be the method of choice (93% vs. 91%). On the other hand, if the aim is exclusively electrical energy production (most common), an abiotic cathode should be preferred since the stable energy production occurs early, and the voltage produced is 398 mV, higher than the 346 mV obtained with the biocathode.

References: Reference numbers according to the order and identification in the main text.