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(Article begins on next page)

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The study of electrochemically active planktonic microbes in microbial fuel cells in relation to different carbon-based anode materials.

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ABSTRACT

Microbial Fuel Cells (MFC) are bio-electrochemical systems that convert chemical energy into electrical energy from the respiratory metabolic profit of electrochemically active bacteria.

In order to contribute to a greater understanding regarding the MFCs performance, real-time quantitative PCR was applied to determining typical planktonic bacteria in the production of electricity in MFCs, evaluating their relations with different carbon-based anode materials: carbon felt (C-FELT), carbon felt with polyaniline (C-PANI) and carbon-coated Berl saddles (C-SADDLES).

Bacteria distribution among the three different MFC anode materials was evaluated: statistically significant differences were detectable for total bacteria ($p < 0.01$), Geobacter ($p < 0.05$) and Shewanella ($p < 0.05$), due to a greater abundance in C-FELT anode MFC. Significant difference ($p < 0.001$) was shown for maximum power density: C-PANI showed a maximum power density of 28.5 W/m3 with respect to C-FELT (4.7 W/m3) and C-SADDLES (4.6 W/m3). In general the largest electrochemically active planktonic microbes was present in the C-FELT while the best carbon-based anode materials results C-PANI.

KEYWORDS: microbial fuel cells; bacterial communities; rt-qPCR; carbon felt; polyaniline deposition; carbon-coated Berl saddles.

1. INTRODUCTION

Microbial fuel cell (MFC) is a bio-electrochemical system that provides a new approach for electricity generation [1]. A typical MFC device is formed by two compartments, one for the anode and the other one for the cathode, which are separated by a cation exchange membrane (CEM). In the anode compartment, organic material is oxidized by microorganisms, thus generating electrons and protons. The electrons are transferred to the cathode compartment through an external electric circuit, meanwhile, the protons flow to the cathode compartment through the membrane [2].

Currently, research on MFCs is thriving and no longer considered as a scientific peculiarity [3] but as a viable future technology [4],[5].

MFCs have recently attracted wide attention as green-energy processes that generate electricity from a variety of organic and inorganic materials [4];,[6]. In particular, MFCs are expected to be applied to the recovery of energy from biomass wastes and wastewater [6],[7],[8], providing dual benefits of wastewater treatment and the production of inexpensive and environmentally friendly energy [2]; also the utilization of marine sediments in MFCs [9],[10] and biosensors [11] are promising applications [5].

It has been the recent discovery of a new metabolic class of electricity-producing microorganisms that has, for the first time, indicated that a wide diversity of organic compounds can be effectively converted to electricity in self-sustaining MFC [12]. These organisms, known as electricigens, degrade (oxidize) organic matter, producing electrons that travel through a series of respiratory enzymes in the cell and make energy for the cell in the form of ATP. The electrons are then released to a terminal electron acceptor (TEA) which accepts the electrons and becomes reduced. Many TEAs such as oxygen, nitrate, sulfate, and others readily diffuse into the cell where they accept electrons forming products that can diffuse out of the cell. However, it is well know that some bacteria can transfer electrons exogeneously (i.e., outside the cell) to a TEA such as a metal oxide like iron oxide. These bacteria called *exoelectrogens* can exogenously transfer electrons producing power in an MFC [13]

The ability of microbes to transfer electrons in the anode can significantly affect the performance of MFCs. Anodic microbial communities were reported to be significantly related with the types of

substrates; for example, Acetobacterium species (sp.), Geobacter sp., and Arcobacter sp. were detected in the anodic biofilm fed with formate [14], Enterobacter sp. was the dominant bacterial species in the MFC with glucose as substrate [15].

A list of microorganism together with their substrates is reported in literature and it is shown in **Table 1**. Marine and river sediment, soil, wastewater, fresh sea-water and activated sludge are all rich sources for these microorganisms [16],[17],[18].

In MFCs, microbes also play crucial roles in energy output and organic contaminants removal [19]. In anodic biofilm, five genera of known exoelectrogens accounted for 23.5% in total communities, including Desulfobulbusm, Geobacter, Desulfovibrio, Pseudononas and Comamonas. The genera of exoelectrogens in planktonic culture included Desulfobulbus, Desulfovibrio, Pseudomonas and Comamonas. Geobacter, an important genus attributing to the power generation in an MFC, was only dominant in the anodic biofilm [20],[21]. Although sulfate reducing bacteria (SRB) was reported to play an important role in transferring electrons on the anode, some groups of SRB that could not form biofilm on the electrode may also reduce sulfate in the planktonic niche [22]. It has already been reported that SRB demonstrate functional dynamics, including electron transfer, sulfate reduction, and converting organic matters, such as acetic and butyric acids to alcohols and acetone via direct electron transfer [23],[24].

The anodic reaction in mediator-less MFCs constructed with metal reducing bacteria belonging primarily to the families of Shewanella, Rhodoferax, and Geobacter consist in transferring electrons to the anode that act as final electron acceptor. Although most of the real mediator-less MFCs are operated with dissimilatory metal reducing microorganisms, an exception was reported with *Clostridium butyricum* [25];[26].

Usually mixed culture MFCs have good performances; using complex mixed cultures (anodic microcosm) allows much wider substrate utilization with respect to pure cultures. In mixed culture MFCs (with anaerobic sludge) there are both electrophiles/anodophiles and groups that use natural mediators together in the same chamber [18].

Over the last decade numerous investigations have been directed to improve performance of an MFC by hanging/modifying the following components: MFC design/architecture/configuration,

electrolytes in solutions, organic fuels, materials and surfaces of electrodes, and electrogenic biofilm on the anode [4]. In particular, the efficiency of electron transfer from electrogens to the anode has been noted to be the most critical factor controlling the overall circuit of an MFC for current production [27],[28];,[29].

In order to improve bacterial adhesion and electron transfer from microorganisms to the electrode several strategies have been developed on carbon-based materials to improve the performances of MFC anode [30],[31]. To address these issues, in this work three different anode electrodes are studied and compared: (1) commercial carbon felt (C-FELT), (2) polyaniline deposited carbon felt (C-PANI) and (3) carbon-coated Berl saddles (C-SADDLES). PANI can be easily deposited on carbon materials and its good conductivity and biocompatibility provide the necessary conditions to be used effectively as surface modifier for electrode materials in the MFCs [32],[33]. Furthermore, the use of carbon-coated Berl saddles have been demonstrated as low-cost solution that satisfy either electrical or bioreactor requirements, increasing the reliability of the MFC processes [34].

The present work is aimed to apply a method for determining typical bacteria in the production of electricity in a MFC by real-time quantitative Polymerase Chain Reaction (rt-qPCR). In order to contribute to a greater understanding regarding the MFCs performance, the purpose of this study was to screening bacteria abundance and to evaluate their relations with different carbon-based anode materials, different physic conditions and time. Three different MFCs were studied with acetate as substrate and carbon felt, carbon felt with polyaniline and carbon-coated Berl saddles as anode materials.

2. MATERIALS AND METHODS

2.1 Materials

Carbon felt, commercial Berl saddles, α-D-glucose (96%), Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$, 98%), Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, 98%) and Potassium ferricyanide ($K_3Fe(CN)_6$, 99%) were purchased from Sigma Aldrich. Seawater was used as inoculum of active microorganisms. The deposition of conductive carbon layer on Berl saddles was performed as reported by Hidalgo and colleagues [34].

131

132 **2.2 MFC configuration and operation**

133 MFC device consists of two circular chambers, i.e. the anode and the cathode. Both compartments
134 were made in Poly(methyl methacrylate) with internal diameter of 12 cm and 1.5 cm of thickness
135 (internal volume for each chamber ~ 170 ml) separated by a cation exchange membrane (CEM, CMI
136 7000, Membranes International Inc., Glen Rock, NJ, USA). Different conductive materials were
137 introduced in each anode chamber: commercial carbon felt (C-FELT) (Soft felt SIGRATHERM GFA5,
138 SGL Carbon, Germany) in MFC1, polyaniline deposited carbon felt (C-PANI) in MFC2 and carbon-
139 coated Berl saddles (C-SADDLES) in MFC3 as anodic materials. For the cathode, it was always
140 used carbon felt (Soft felt SIGRATHERM GFA5, SGL Carbon, Germany) as electrode material. Each
141 conductive material was connected with a graphite rod (5 mm in diameter) to ensure an effective
142 current transport. MFCs were inoculated in the anode chamber by sea water (Arma di Taggia, Italy),
143 previously enriched with following cultures (in five steps) in anaerobic conditions (V inoculum 10%
144 of synthetic substrate previously described). The first 10 days of tests have been conducted in Open
145 Circuit Voltage condition, in order to have the adaptation of bacteria at the new conditions inside the
146 MFCs. After the start-up period, MFCs were operated under external resistance of 1000 Ω.

147 All the investigations were carried out under the same operational conditions, in fed-batch mode by
148 using a multi-programmable syringe-pump (NE-1600, New Era Pump System) at room temperature
149 22 ± 2 °C. The mixing of the solutions at both anode and cathode chambers was obtained by
150 recirculating anolyte and catholyte from a 500 ml reservoir, respectively at a high flow rate (30
151 mL/min) by multichannel peristaltic pumps at both anode and cathode chambers (Peri-Star Pro 4
152 and 8 channel, USA, respectively).

153 Evaluations were performed over a period of 5 weeks feeding in the anodic solution sodium acetate
154 (1 g/L) as synthetic substrate and peptone (1.25 g/L) as nutrient sources for microorganisms growth.
155 The cathodic compartment was filled by potassiumferricyanide (6.58 g/L) used as oxidant compound.
156 To prepare anodic and cathodic solutions, a buffer of inorganic salts, i.e. $Na_2HPO_4 \cdot 2H_2O$ (8.2 g/L)
157 and $NaH_2PO_4 \cdot H_2O$ (5.2 g/L) was used.

158

159 **2.3 Electrochemical measurements and analyses**

160 The comprehension of the MFCs trend during time-course of tests required the exploration of both
161 physiological and electrochemical parameters. For this reason, every 2-3 days, pH, Redox Potential
162 (rH), conductivity and optical density (OD) at 600nm were monitored taking planktonic samples from
163 liquid samples. Moreover, electrochemical characterizations, including Open Circuit Voltage (OCV),
164 Linear Sweep Voltammetry (LSV) and Current Interrupt (CI) method, were conducted under 1000 Ω
165 external resistance from day 3 to day 35, allowed to understand the system from electrical and
166 biological point of view. Electrochemical experiments were performed on a multi-channel VSP
167 potentiostat/galvanostat produced by BioLogic. Measurements were recorded by using EC-Lab
168 software version 10.1x (BioLogic) for data acquisition. All tests were carried out using a two electrode
169 setup, where the working electrode was coupled to the anode and both counter electrode and
170 reference electrode were connected to the cathode. Polarization curves were performed at a scan
171 rate of 1mV/s when a constant OCV was achieved, from the open-circuit cell voltage V_0 (where I =
172 0) to the short-circuit cell voltage Vsc = 0 (where I = I_{max}). From the I-V curves, the power density
173 was calculated by P = IV/*v*, where I, V and *v* represent current, recorded voltage output and the total
174 anode compartment (TAC), respectively.

175 CI measurement was performed to determine the ohmic resistance of the MFC through the
176 interruption of the current flow and the resulting voltage transients [4]. The circuit was opened
177 causing a steep potential followed of further low rise. The resistance was evaluated as $R_\Omega = V_R/I_0$
178 and a mean value of different tests were computed [34].

179 Furthermore, both anode potential (versus Ag/AgCl electrode) and MFC potential, were maintained
180 in OCV conditions during the first 3 days of operation and then an external resistance of 1000 Ω was
181 connected from 3 to 35 days, which allowed continuously recorded the voltage generated every 60
182 s during the whole time-course of tests by using a Data Acquisition Unit (Agilent, 34972A). These
183 voltage data were used to calculate current and therefore power density according to the external
184 resistance applied and the internal volume of anode chamber of MFCs. Even if the tests were
185 conducted using buffer solution in both chambers, a pH adjustment by HCl 0.01M was necessary

186 because the anode pH tends to increases, probably related to the increase of conductivity of anode
187 solution due to the use of a medium containing sea-water.

188 **2.4 Biological analysis**

189 On the basis of literature were selected procedures in order to analyze the bacterial communities
190 involved in anaerobic fermentation leading to production of electricity in a MFC [5];[35];[36];[37];[38].
191 The rt-qPCR analysis were performed for the following genera of microorganisms: Total Bacteria,
192 Total Sulfate Reducing Bacteria (SRB), Acetobacter (Gram-), Clostridium (Gram+), Geobacter
193 (Gram-), Saccharomyces (Yeast), Shewanella (Gram-). Gene target primers for each strain were
194 selected by the international scientific literature (**Table 2**) and they have been tested in their
195 functionality: a specific rt-qPCR protocol was used for each primer, then a new common rt-qPCR
196 protocol was developed in order to optimize and standardize the analysis at the same time.

197 **2.5 DNA extraction and purification**

198 In order to evaluate microbial community variability of in MFC, 1.8 ml of culture media from MFC
199 were centrifuge at 10000 g for 30 sec, and supernatant was discarded. The DNA extraction was
200 performed with a commercial kit (UltraCleanTM Microbial DNA Isolation Kit, MO-BIO Laboratories
201 Inc., Carlsbad, CA).

202 An electrophoresis gel on 2% agarose and 1X TBE buffer (1L, 10X TBE buffer stock: 121.1 g Trizma
203 base, 61.8 g boric acid and 7.4 g EDTA) was performed after each extraction to check genomic DNA
204 integrity. 20 µl of DNA sample (1:10) with 20 µl of loading buffer (950 µl of glycerol 30%, 50 µl of
205 bromophenol blue) and 15 µl of ethidium bromide were loaded into the gel together with a molecular
206 mass marker (M). The gel was placed in an electrophoresis chamber, which was then connected to
207 a power source. The applied electric current is 100 V for 70 min.

208 **2.6 DNA quantification**

209 The fluorimetric quantification of each DNA sample was performed using QubitTM Fluorometer and
210 QubitTM dsDNA HS Assay by Invitrogen (distributed by Life Technology Ltd. – Paisley, UK), taking
211 into account the manufacturer's instructions.

212 Samples with a concentration greater than 1 µg/mL and showing sufficient DNA quality as observed
213 by gel electrophoresis were used for the analysis. These restrictions were applied because of

214 difficulty in quantifying poor quality DNA or small concentration of it. Samples were stored at -20°C
215 until performing RT-qPCR analysis.

216 **2.7 RT-qPCR**

217 After DNA extraction and purification RT-qPCR was used to identify and quantify microorganisms.
218 Each RT-qPCR was performed in a 96 wells plates. All RT-qPCR were carried out in a 20 µl volume,
219 in singleplex, with specific primer; each sample was tested in triplicate.
220 The reactions used a standard super-mix (Bio-Rad SsoFast_EvaGreen SuperMix) and the RT-qPCR
221 Chromo4 (Bio-Rad) with Opticon Monitor 3 Software.
222 After that RT-qPCR protocols were tested with their specific primer
223 [39],[40],[41],[42],[43],[44],[45],[46],[47],[48], a new common thermal protocol for RT-qPCR was drawn
224 and tested. Reaction conditions were as follow: 1° step: 95°C for 3.5 minutes, as initial denaturation
225 phase; 2° step: 95°C for 30 sec, as denaturation phase; 3° step: 55°C for 45 seconds, as annealing
226 phase; 4° step: 72°C for 30 sec, as extension phase; 5° step: Reading plate. After 40 cycles (steps
227 2°-5°) a melting curve, was run with the following thermal conditions: from 55 °C to 95 °C read every
228 0.5 °C, and read plate at 95 °C.
229 To quantify the Total Bacteria the reaction use standard power-mix (Bio-Rad IQTM Multiplex
230 PowerMix) and the RT-qPCR Chromo4 (Bio-Rad) with Opticon Monitor 3 Software. The RT-qPCR
231 thermal protocol was the same (melting curve was not performed).
232 The standard curves had six points and were calculated according to the threshold cycle method.
233 There was a 1:10 dilution factor between each standard curve point (range 10 – 10^6). The standards
234 and samples were tested in triplicate. The triplicate value was accepted only if the coefficient of
235 variation was below 20%. The correlation coefficient was considered sufficient if above 0.980. The
236 PCR efficiency for the different strains was between 79% and 104%. We used 2 µl of a 1:10 dilution
237 factor of each DNA extract sample for the amplification. This quantity was evaluated as the best
238 among various tested quantities according to a standard curve and acceptable PCR efficiency. The
239 1:10 dilution limited the effect of inhibitory substances present in this kind of sample.
240 In order to obtain an absolute quantification of bacteria in MFC samples, a standard curve of specific
241 genomic DNA was performed, in this way, the quantification of bacteria could be expressed in terms

242 of gene copies number as a useful and necessary parameter to compare different samples (**Table**
243 **2**). After each PCR analysis, in order to confirm fragment amplification, gel electrophoresis on 2%
244 agarose was performed, with the technical procedure previously described for evaluation of DNA
245 integrity.

246 **2.8 Statistics**

247 Statistical analyses were performed with the SPSS Package version 21.0 for Windows. Student's t-
248 test and one-way analysis of variance (ANOVA) were applied to compare two or more groups of
249 independent samples, respectively, while Spearman's correlation was used to test for possible
250 associations between the variables. For ANOVA testing, the homogeneity of the variance was firstly
251 assessed through the Levene test, thus the equal variance of Tukey's test was assumed for post
252 hoc multiple comparisons. The differences and correlations were considered significant at $p < 0.05$.
253

254 **3. RESULTS AND DISCUSSION**

255 **3.1 MFCs electrochemical measurements and analyses**

256 **Figure 1** is an overview of the electrical behavior of the three MFCs. The first three days, MFCs
257 were maintained in OCV conditions, in order to reach stable voltage conditions of potential and to
258 favorite bacterial adaptation and growth and without the stress induced by external resistances.
259 **Figure 1** showed many fluctuations mainly due to the fed-batch condition. Changing from OCV to
260 1000 Ω resistances provokes a drop of voltage, more evident (about 50% of OCV) the first week
261 where the biofilm probably was not so stable. Moreover, it was possible to observe that MFC 1 which
262 operated using C-FELT showed more evident fluctuations possibly due to both external resistance
263 connection and fed-batch feeding of substrate. Furthermore, MFC 2 which operated using C-PANI
264 showed more stable electrical conditions, probably due to the PANI deposition improved the bacterial
265 adhesion on felt facilitating electron transfer from microorganisms to the electrode. Similar results
266 were also obtained in MFC 3 confirm that C-SADDLES satisfied the electrical requirements in MFC.
267 The complete comprehension of the MFCs trend during time-course of test required the exploration
268 of both physiological and electrochemical parameters, as shown in **Table 3**.

269 Initial pH was 6.65 ± 0.02 for the three cells. It sharply increases for the three cells during the first
270 15 days till 8.2, 8.3 and 8, respectively for MFC 1, 2 and 3. After that period the measured pH values
271 (referred before adjustment) were constant in the range of 7.5 - 8.3. Redox Potential (rH) rapidly
272 decreased in the first 3 days (OCV conditions) from values near to 0 ± 3.6mV at time 0 to negative
273 values of -120 ± 30 mV as average values for the three cells at time 3 days. The rH data confirmed
274 that there were reducing conditions and hence the good condition of electron release in the liquid
275 medium, thanks to metabolic activity of microorganisms starting from sodium acetate, as electron
276 donor. The low rH was also a demonstration of anaerobic condition establishment, necessary to
277 recovery electrons on anode electrodes. Moreover it was in agreement with Optical Density (OD)
278 measures, because in the first three days an exponential phase of total cell were noted in the three
279 MFCs: after a lag-phase lesser than 1 day in which microorganisms reorganize their molecular
280 constituent to adapt to a new environmental conditions, the metabolism starts releasing reduced
281 compounds, such as liquid and gas metabolites beyond protons and electrons, as confirmed from
282 electrochemical analysis [49]. The anolyte conductivities shows a linear increase of about 7 units
283 (from 27 to 34 mS/cm). These highly conductivity values (bigger than those ones present in real
284 wastewater, typically in order of only 1 mS/cm [50], are essentially due to the consecutive addition
285 of phosphate buffer and sea water in the anode feeding, useful mainly for charge transfer (electrons
286 and cations, towards anode-electrode and CEM membrane, respectively), approximately neutral pH
287 maintenance, and for supply micronutrients for bacteria growth.
288 In **Figure 1** the voltage of MFCs increased from low to high values, resulting in a greater power after
289 3 days from the beginning of the experiments.
290 Moreover in the first 20 days there were a sharply decrease of voltage, these decreases were
291 probably due to an imbalanced ratio between carbon and nitrogen (C/N= 100); after day 19th it was
292 established a ratio C/N=30 adding peptone and the voltage increased.
293 Polarization curves (LSV) describes voltage as a function of current representing a powerful tool for
294 a rapid analysis and characterization of MFC during tests (**Table 3**). They were checked at initial
295 time, the 3rd day, before to put 1000 Ohm resistances, and day 32 before to stop tests. LSV curves
296 demonstrate a great increase of MFC performances from the starting point: in particular MFC 2

297 (carbon felt with PANI deposition) shows the biggest power density value of 35 W/m^3, one order of
298 magnitude more than MFC1 and MFC2. MFC3, with 3-D granular electrode shows a curve very
299 different from both MFC1 and MFC2, showing always a constant behavior from OCV till Short Circuit
300 Current conditions (SCC). Maximum power density for MFC3 was obtained at the end of experiment,
301 reaching 4.6 W/m^3 (**Figure 2**). Similar behavior of polarization curves has been obtained in other
302 works with graphite in granules as electrode [51] [52].
303 Even if MFC2 (with PANI deposition) gave the highest power density by LSV, it does not completely
304 reflect the real behavior under load of 1000 Ohm of about 2.5 W/m^3. MFC2 has the most stable and
305 highest value of voltage than MFC1 and MFC3, but with the same order of magnitude. On the
306 contrary, the response of the voltage slop of 1 mV/s by LSV showed MFC performances one order
307 of magnitudes higher than MFC1 and MFC3, probably disregard the affect of bacteria adaptation.
308 Author's opinion is that LSV slightly overestimated electrical parameters (I, V) and therefore power
309 density, while 1000 Ohm resistances did not fit the best resistance in order to have maximum power
310 point (MPP) that were one order less for MFC1 and MFC3 and two orders less for MFC2.
311 **Figure 3** evidences an important increase of the maximum power density of the MFC2 (28.5 W/m^3)
312 with respect to MFC1 (4.7 W/m^3) and MFC3 (4.6 W/m^3) after 35 days of operation, which can be
313 related to an important reduction of the ohmic resistance of the cell by using C-PANI. Results
314 confirmed that PANI effectively increases the conductivity of the material favoring the recovery of
315 electrons and increasing the sustainability of the process. In addition, **Figure 4** shows that despite
316 of the higher internal resistance of MFC3 with respect to MFC1, similar results were obtained for
317 both cells suggesting that microorganisms were effectively adapted to the new anode material which
318 could be advantageous for continues MFC applications.

319 **3.2 RT-qPCR and target microbial populations in MFCs**

320 The fluorimetric quantification of DNA samples ranged between 25.4 and 128.0 µg/mL_and all
321 samples had intact DNA.
322 The realization of a unitary RT-qPCR protocol proved to be valid for all microorganisms selected
323 either bacteria or yeasts. The method gives highly reliable results: standard curve R^2 is never lower
324 than 0.984. This allowed to carry out standardized analyses and to compare different samples.

325 RT-qPCR analyses have highlighted that all the different microbial populations were present and
326 quantifiable in each MFC, with the exception of Acetobacter that resulted undetectable in all the
327 samples probably owing to maintenance of the anaerobic conditions; acetic acid bacteria are
328 characterized by aerobic metabolism with incomplete oxidation of organic substrates.
329 The detected level of various bacteria groups is displayed in **Figure 4**. Groups varied in quantity
330 during the MFC operation and were always present at all, total SBR resulted prevalent followed by
331 Shewanella. In this study were analyzed bacteria belonging to Proteobacteria (Acetobcter, α-
332 Proteobacteria; Desulfovibrio and Geobacter, δ-Proteobacteria; Shewanella, γ-Proteobacteria),
333 Firmicutes (Clostridium) and Ascomycota (Saccharomyces) phyla.
334 As described in the literature, in MFC planktonic culture are present mainly bacteria belonging to the
335 Proteobacteria phylum [1]·[24]; β- and γ- Proteobacteria resulted predominant among the
336 Proteobacteria phylum in the Sun et al. (2010) instead Wang et al. (2014) showed a majority of δ-
337 and β- Proteobacteria.
338 The selected typical bacteria accounted for 22% of total Bacteria and the percentage of each
339 microorganisms strain was variable (**Figure 4**); RT-qPCR analyses have identified 16 % (range:
340 5.35% – 36.65%) SRB, 3.6 % (range: 1.62% – 7.24%) Shewanella, 2.4 % (range: 0.02% - 8.76%)
341 Geobacter, 0.001 % (range: 0.000% - 0.003%) Saccharomyces, 0.001 % (range: 0.0001% -
342 0.0039%) Clostridium and Acetobacter resulted absent. SRB resulted the most abundant
343 microorganisms among total bacteria and always in higher quantity than other species; a similar
344 datum was also present in a research conducted by Logan using MFCs containing sediments [13].
345 To date more than 220 species belonging to 60 different genera of SRB have been described [53]
346 this might justify the found percentages.

347 **3.3 MFCs considerations**

348 The differences among the three anode materials were evaluated comparing the performance of the
349 three MFCs. Considering carbon felt and carbon felt with PANI (MFC1 vs MFC2), no statistically
350 significant differences resulted for total bacteria (t-test, $p > 0.05$) although in MFC1 they decreased
351 steadily by one order of magnitude (from 10^9 to 10^8), whereas in MFC2 they kept constant all trial
352 long. No statistically significant differences resulted for SRB (t-test, $p > 0.05$). Although Clostridium

353 decreased in MFC1 and keep constant in MFC2, there were no statistically significant differences (t-
354 test, $p > 0.05$). During the trial in both of the MFCs Geobacter increased, contrariwise
355 Saccharomyces decreased and Shewanella remained constant: the differences were not statistically
356 significant for all the three (t-test, $p > 0.05$).
357 The MFCs system utilized in this test provided performance in line with the scientific literature
358 [54]·[55]·[56]: maximum power density reached is 255.7 W/m^3 and maximum current density reached
359 is 2138.5 A/m^3. The MFC2 configuration gave the best performance: the use of PANI increases in a
360 statistically significant manner the performance of the cell. In the future could be useful to measure
361 the substrate concentration to quantify more accurately the performance of the system.
362 Power density and maximum current showed statistically significant differences (t-test, $p < 0.001$ and
363 $p = 0.001$): MFC2, that presented the deposition of PANI, has higher value of one order of magnitude;
364 therefore it was possible to state that deposition of PANI improved the performance of the cell.
365 Correlation between amount of microorganisms and power density were not detectable because of
366 reduced availability of data.
367 Considering carbon felt and graphitized Berl saddles (MFC1 vs MFC3), the analyses highlighted that
368 the quantity of microorganism was always higher in MFC1: this could be due to sampling of
369 planktonic component rather than anode biofilm, since Berl saddles provide greater adhesion surface
370 to the microorganism. Total bacteria and SRB decreased after some day of increase in MFC1,
371 contrariwise they kept constant in MFC3: statistically significant differences resulted only for total
372 bacteria (t-test, $p = 0.011$) but not for SRB (t-test, $p > 0.05$). No statistically significant differences
373 resulted for Geobacter (t-test, $p > 0.05$), that increased in both of the cells since the twelfth day.
374 Shewanella decreased slightly in MFC1, while remained constant in MFC3 (t-test, $p = 0.014$).
375 Maximum power density is approximately equivalent in both of the MFCs, contrariwise the maximum
376 current is higher in MFC1 although in no statistically significant way (t-test, $p > 0.05$).
377 Total bacteria seemed to correlate positively with the maximum current, but this was not verifiable
378 with statistical analysis because of reduced amount of data.
379 One-way analysis of variance (ANOVA) was applied to evaluate differences in the distribution of
380 bacteria among the three different MFCs. In general statistically significant differences were

381 detectable for total bacteria ($p = 0.007$), Geobacter ($p = 0.050$), Shewanella ($p = 0.046$), current
382 density ($p < 0.001$) and power density ($p < 0.001$). Post hoc test (Tuckey) showed significant
383 differences in the anodic chambers: - for the total bacteria in relation to carbon felt with or without
384 PANI ($p = 0.047$) and carbon felt or graphitized Berl cells ($p = 0.006$) these results were due to a
385 larger number of total bacteria in the MFC1 during the tests; - for Shewanella in relation to carbon
386 felt or graphitized Berl cells ($p = 0.037$): in the MFC1 there was a greater presence of Shewanella
387 compared to MFC3, contrariwise mean presence of Shewanella was similar in anodic chamber with
388 or without PANI. Otherwise Post hoc test (Tuckey) showed significant difference for maximum power
389 density in relation to - carbon felt with or without PANI ($p < 0.001$), - carbon felt with PANI or
390 graphitized Berl cells ($p < 0.001$): these differences were due to a higher power density of a MFC2;
391 for maximum current density in relation to - carbon felt with or without PANI ($p < 0.001$), - carbon felt
392 with PANI or graphitized Berl cells ($p < 0.001$): also in these cases the differences were due to
393 influence of MFC2, that had a major current density.

394 **4. Conclusion**

395 RT-qPCR method proposed herein is a useful tool for quantify typical electrochemically active
396 planktonic microbes and their differences in relation to different MFC carbon-based anode materials,
397 nevertheless our results cannot yet clearly characterize the whole active populations in a MFC.
398 In general the best carbon-based anode materials results carbon felt with PANI deposition. PANI
399 deposition, having molecular structures that resemble electron mediators, may function as mediators
400 that improve electron transfer [57]. It has proven to enhance the MFC performance considerably and
401 to decrease output voltage fluctuation under external load. Anyway, despite the advances made by
402 electrode materials (from our study and other researchers) significant hurdles remains before render
403 MFC technology ready for the practical deployment in either wastewater treatment or power
404 generation beyond small sensors. Moreover, further studies are needed to characterize the whole
405 electrochemically active microbial community and highlight differences between the anode-attached
406 and planktonic communities, so to understand the relation within the microbial communities and
407 increase the performance of the system.
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409

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597 **Table 1**. Microbes used in MFCs: microorganism and substrates (modified by Du et al., 2007)

598

Microbes	Substrate	Applications	Ref.
Clostridium beijerinckii	Starch, glucose, lactate, molasses	Fermentative bacterium	[58]
Clostridium butyricum	Starch, glucose, lactate, molasses	Fermentative bacterium	[26,58]
Desulfovibrio desulfuricans	Sucrose	Sulphate/sulphide as mediator	[59], [60]
Geobacter metallireducens	Acetate	Mediator-less MFC	[61]
Geobacter sulfurreducens	Acetate	Mediator-less MFC	[20] [62]
Gluconobacter oxydans	Glucose	Mediator (HNQ, resazurin or thionine) needed	[63]
Shewanella oneidensis	Lactate	Anthraquinone-2,6-disulfonate (AQDS) as mediator	[64]
Shewanella putrefaciens	Lactate, Pyruvate, Acetate, Glucose	Mediator-less MFC; but incorporating an electron mediator like Mn (IV) or NR into the anode enhanced the electricity production	[65], [66],[67]
Acetobacter aceti	Glucose	Mediator-less MFC	[68]
Gluconobacter roseus	Glucose	Mediator-less MFC	[68]
Saccharomyces cerevisiae	Artificial Wastewater	methylene blue (as indicator)	[69]
Clostridium thermohydrosulfuricum	Artificial Wastewater	methylene blue (as indicator)	[69]

599 **Table 2:** Microorganisms, Primers, Genomic Standards and total number of bases in the genomic

600 DNA (bp), genes and number of copies of gene in µL of solution, tested in MFC biological analysis.

Microorganism	**Primer name (gene target)** (5' --> 3')	**Genomic Standard (ATCC code)** **N. bases (bp)**	**Gene bp** **N. gene copies/µL**	**Ref.**
Acetobacter	Ace F: CGCAAGGGACCTCTAACACA Ace R: ACCTGATGGCAACTAAAGATAGGG	*Acetobacter diazotrophicus* (49037D-5) 4,00E+06	110 1,52E+06	[48]
Total Bacteria	16S RNA F: AGAGTTTGATCMTGGCTCAG 16S RNA R: TTACCGCGGCKGCTGGCAC Probe: CCAKACTCCTACGGGAGGCAGCAG	*Desulfovibrio vulgaris* (29579D-5) 3,57E+06	About 600 8,52E+06	[42]
Clostridium	Clo F: ATTAGGAGGAACACCAGTTG Clo R: AGGAGATGTCATTGGGATGT	*Clostridium difficile* (9689D-5) 4,18E+06	307 1,45E+06	[47]
Geobacter	Geo F: AAGCGTTGTTGTTCGGAWTTAT Geo R: GGCACTGCAGGGGTCAATA	*Geobacter metallireducens* (53774D-5) 4,01E+06	313 2,27E+07	[39]
Saccharomyces	Sac F: GCGGTAATTCCAGCTCCAATAG Sac R: GCCACAAGGACTCAAGGTTAG	*Saccharomyces cerevisiae* (9763D) 1,21E+07	151 3,77E+07	[48]
Shewanella	She F: GCCTAGGGATCTGCCCAGTCG She R: CTAGGTTCATCCAATCGCG	*Shewanella oneidensis* (700550D) 5,13E+06	108 1,19E+07	[44]
Total SRB	AprA F: GGGYCTKTCCGCYATCAAYAC AprA R: ATCATGATCTGCCAGCGGCCGGA	*Desulfovibrio vulgaris* (29579D-5) 3,57E+06	About 300 8,52E+06	[41] [43] [45] [46]

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602

603 **Table 3**. MFCs physiological and electrochemical parameters.

Parameters	MFC1	MFC2	MFC3
pH (average value)	7.8 ± 0.5	7.6 ± 0.4	7.59 ± 0.5
Conductivity (mS)	31.4 ± 2.9	30.7 ± 2.4	29.9 ± 2.7
Redox Potential (mV)	-60 ± 33	-86 ± 40	-95 ± 43
P max (W/m^3)	4.8	28.1	4.6
P max (W/m^2)	0.074	0.430	0.071
OCV (V)	0.78	0.73	0.70
I_{scc} (A/m^3)	35.5	155.5	24.3
$P_{average}$ (W/m^3) under 1000 Ohm	1.70 ± 0.68	2.40 ± 0.19	1.61 ± 0.24

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622 **FIGURE CAPTIONS**

623 **Figure 1.** Voltage vs time course of test from time 0 to 35 days a), current densities according to
624 total anode volume b) and power density c) from MFC1, MFC2 and MFC3.

625

626 **Figure 2.** Polarization curves on the left and corresponding power on the right of MFC1, MFC2 and
627 MFC3, respectively.

628

629 **Figure 3**. MFCs power density (P) and ohmic resistance (R).

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631 **Figure 4.** Radar graph of gene copies/mL values (logarithmic scale) of samples from MFC1, MFC2,
632 MFC3 for each strain by each probe during the time (from 1st to 5th week)..

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