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Monitoring microbial communities' dynamics during the start-up of microbial fuel cells by high-throughput screening techniques

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ABSTRACT

Microbial Electrochemical Technologies are based on the use of electrochemically active microorganisms that can carry out extracellular electron transfer to an electrode while they are oxidizing the organic compounds. The dynamics and changes of the bacterial community in the anode biofilm and planktonic broth of an acetate fed batch single chamber air cathode MFC have been studied by combing flow-cytometry and Illumina sequencing techniques. At the beginning of the test, from 0 h to 70 h, microbial planktonic communities changed from four groups to two groups, as revealed by DNA content, and from three groups to one group based on the cell membrane polarization revealed by a $DiOC_6(3)$ probe. Between 4th day and 13th day, microbial communities changed from one group to a maximum of three groups, monitoring DNA content, and from one group to two based on the cell membrane polarization. The *16S rDNA* gene profiling confirmed the shift in microbial communities, with *Acinetobacter* (39.34%), *Azospirillum* (27.66%), *Arcobacter* (4.17%) and *Comamonas* (2.62%) being the most abundant genera at the beginning of MFC activation. After 70 h the main genera detected were *Azospirillum* (46.42%), *Acinetobacter* (34.66%), *Enterococcus* (2.32%), *Dysgonomonas* (2.14%). Data obtained have shown that flow cytometry and illumina sequencing are useful tools to monitor "online" the changes in microbial communities during the MFCs start-up and the increase of *Azospirillum* and *Acinetobacter* genera is in good agreement with the MFC voltage generation. Moreover, monitoring planktonic populations, instead of the less accessible anode biofilm, was in good agreement with the evolution of MFC voltage.

1. Introduction

Microbial Electrochemical Technologies (METs) promise the prospect of producing energy or bio-based compounds from bio-wastes [1,2]. METs share the principle of microbial catalyzed anodic substrate oxidation and can be addressed to various applications such as: electricity production (microbial fuel cells; MFCs) (Liu et al., 2014; [3]), hydrogen production (microbial electrolysis cells, MEC) [4], bioremediation (option for nitrate removal operating either in MFC or in MEC mode) [5] or bio-electro synthesis (reduction of CO_2 in high value products) [6,2,7].

METs are based on a bio-electrochemical process that converts the chemical energy of biodegradable organic compounds into electricity or high value chemicals by bacterial metabolism [8]. METs are based on the use of these electrochemically active microorganisms, which can transfer extracellular electrons to an electrode (anode) while they are oxidizing (and thus removing) organic compounds [9–12]. The electrons can be transported to the electrode by several mechanisms, better known as extracellular electron transport (EET) mechanisms [9,13]; Sidow et al., 2014; [14,15]. Two kinds of pathways of EET are currently assumed to be used by microorganisms. Direct electron transport (DET) requires physical contact between a microbial cell membrane or membrane organelle, and the anode electrode surface, excluding the presence in the media of any diffusional redox chemical species [9,16]. C-type cytochromes associated with outer membranes and conductive pili are involved in this process [17–191,20,21]. DET is allowed by cell contact with the extracellular electron acceptor/donor system, and in METs, efficient DET depends on the existence of a biofilm or at least a single cell layer on the electrode surface [22,23].

The second EET method normally used by microorganisms is defined as mediated electron transfer (MET). These microorganisms such as *S. oneidensis* or *Pseudomonas aeruginosa*, need redox mediators to carry out indirect electron transfer to the electrode; a

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mediator is a molecule (shuttle) that can be regenerated and enables the electron transfer between the external electron donor/acceptor and the microorganism even at longer distances [1,8,20]. Different kinds of mediators such as phenazine, flavin, 2-amino3-carboxy1,4 naphthoquinone can be used during MET by a wide range of microorganisms [1,20]. As described previously, the bacteria responsible for the process are specific bacterial species, called electroactive bacteria (EAB), mainly belonging to the Alpha-, Beta-, Delta- and Gamma-subgroups of Proteobacteria [24,25]. In any case, not all the microbes present in the biofilm consortium are involved in the EET process; nevertheless, they can be involved in other functions such as providing organic nutrients to the electrogenic microbes of the consortium [26]. Therefore, EAB with biofilm formation properties are of great interest for MET applications. Understanding and exploiting the bacterial composition of the biofilm and monitoring how these species interact during the electron transfer process can be a useful way to develop microbial electrochemical technologies. Several methods to describe the bacterial electron transfer biofilm are reported in the literature [27]. Among these methods, in vivo studies can be a useful way to describe and monitor the complexity of an EAB biofilm such as the planktonic bacterial community [28]. Flow cytometry (FC) is a high-throughput method to describe and analyse the optical characteristics of the cell. Commonly FC is used for medical research or biological analysis while it is less common in microbial cell studies. Therefore, the use of FC to describe microbial communities and the dynamic of these communities during microbial processes is still rare in the literature [29,28]. FC techniques can be used to monitor and detect changes in the structure of microbial communities [28]. The important advantage of using flow cytometry is the possibility of analysing a huge amount of cells in a very short time (about 1000 bacteria s^{-1}), which allows the gathering of larger sets of data with more precise information, in comparison with conventional microbiology methods (such as staining, followed by microscope cell counting) used to monitor microbial populations [30]. Moreover, through FC it is possible to study bioprocesses in depth because it allows analysing in real time the microbial cell responses to different external parameters. Harnisch et al. [29] have already reported the application of FC to characterize the anode biofilm of a bio electrochemical system and in particular the influence of pH in the anode biofilm composition. Hewitt et al. [31] have reported how to describe the physiological state of *Escherichia coli* using multi-staining parameter flow cytometry. As previously described, membrane polarization may change due to an external stimulus or during EET. Fluorescent dyes (such as $DiOC_6(3)$ or Propidium iodide) in combination with flow cytometry analysis can be used to determine and detect the whole and the polarized cell membranes [30]: in fact, the oxidation/reduction reactions that occur between the substrate and the final electron acceptor for power generation during an EET process, influence the microbial electrochemical gradient membrane. Moving beyond flow cytometry, high throughput sequencing methods, also called “next generation sequencing” have provided a great quantity of information on microbial ecology and at the same time, sequencing costs have rapidly decreased [32–34]. Owing to their high throughput and the decreasing cost per sequence, next generation sequencing techniques have great potential to describe the diversity and composition of microbial communities in all microbiological systems such as METs [32,34]. In fact, Illumina technology can generate in a single run millions of amplicon sequences, thus providing high coverage for metagenomic studies of microbial communities [33]. For this reason, this technology can be used to improve the current knowledge of microbial community structure involved in EET mechanisms [32,34]. Starting from these points, the aim of this study was to evaluate the physiological status of the EAB of the anode biofilm and of the planktonic liquid of a single chamber air cathode microbial fuel cell. To carry out this study, FC and fluorescent dyes’ staining were combined together to monitor in real time the changes in the EAB community during the start-up and the stable phase of current production of a microbial fuel cell. Through these techniques the analyses conducted were used mainly to monitor the total number of bacterial cells (Syb), the cells with polarized membranes ($DiOC_6(3)$) and the cells with whole membranes. Moreover, in order to characterize the planktonic and anode biofilm microbiomes, Illumina high-throughput sequencing was used. The combination of high-throughput (flow cytometry and Illumina sequencing) techniques used in this study can increase understanding of the mechanisms involved in the dynamic of biofilm and planktonic EAB selection during the METs bioreactors’ acclimation and during the stable phase of current production.

2. Materials and method

2.1. Microbial fuel cell reactors

A single-chamber air-cathode MFC was constructed as previously described (Pepè Sciarria et al., 2014; [35]). The reactor consisted of a Plexiglas liquid chamber (4 cm x 5 cm), with a volume of 28 mL; a graphite fiber brush was used as anode with titanium wire as core (Panex 33 160 K, Zoltek), 2.5 cm in both outer diameter and length. The estimated surface area of the anode was of 0.22 m^2 or 18,200 $m^2\ m^{-3}$ brush-volume (95% porosity) [8]. The cathode (diameter of 3.8 cm) was made as previously described [36]. Anode and cathode were connected with copper wire; the voltage across an external resistor of 1 kΩ, was monitored every 15 min using a multimeter (2700, Keithley, United States) connected to a personal computer. Polarization and power density curves were obtained by changing the external resistance (0–10 kΩ) every 30 min and measuring the cell voltage for each different resistance. The MFC reactor was first acclimated by using the wastewater from an urban wastewater treatment plant (Peschiera Borromeo, Italy). The wastewater was enriched with a medium (Na_2HPO_4 9.152 g L^{-1}, $NaH_2PO_4{\cdot}H_2O$ 4.904 g L^{-1}, NH_4Cl 0.62 g L^{-1}, KCl 0.26 g L^{-1}) containing 1 g L^{-1} of sodium acetate, trace minerals solution and vitamins solution (982.5 mL medium, 12.5 mL trace minerals, 5 mL vitamins for each liter) [37]. The initial pH of the solution was 7.0 ± 0.2. The cell was considered acclimated when the maximum voltage output reached ≈500 mV for three consecutive cycles. Feed solution was replaced when the voltage decreased below ≈50 mV, forming one complete cycle of operation.

A single chamber air cathode MFC was run for 11 days in batch mode until a stable voltage production was obtained. Samples from the anode and from the planktonic phase were collected from the beginning of the start-up, i.e. at the inoculation of the MFC and until the stable state voltage production. In particular, anode samples were collected by cutting a portion of graphite fiber by using a sterilized scissor [38] while planktonic liquid samples were collected using a micropipette equipped with sterilized tips (V = 50 μl for each sample). All the samples collected were analysed by flow cytometry to obtain an on-line dynamic of the bacterial population while some of them were also analysed by *16S rRNA* gene profiling to describe the microbial population dynamics during voltage generation.

2.2. Microbial population analysis

The microbial fuel cell was studied (at room temperature 23 ± 1 °C) during the initial stages of the biological process (from 0 to 11 days) by using two screening techniques: flow-cytometry and Illumina sequencing. Microbial population dynamics and the

evolution of an electroactive biofilm over the anode (and planktonic phase) were investigated by flow cytometry (Becton Dickinson Accuri C6, USA) quantifying all microbial populations using SYBR-Green I staining. SYBR-Green I, permeates the membrane of total cells and stains the nucleic acids with green fluorescence. In addition, microbial populations were analysed by measuring the level of cells' membrane polarization using the $DiOC_6$ probes. $DiOC_6$ is a membrane-potential-sensitive probe, which labels polarized cell membranes [39]. Therefore, the SYBR-Green staining was used to enumerate the total microbial population, and the $DiOC_6$ staining to identify the metabolically-active microbial cells. SYBR-Green I and $DiOC_6$ fluorescence were reported versus the Forward Scatter (FSC), the parameters this latter providing useful information on cell size. Total genomic DNA was extracted using a phenol/chloroform method [40]. DNA extracted was quantified using QuantiFluor® dsDNA System (Promega, USA). Six samples (DNA extraction >5ug/ml) were chosen for Illumina sequencing (Istituto Italiano di Tecnologia, Genova). Reads were trimmed and filtered by FASTQX and the resulted high quality reads were managed by the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.0 [41] with the GreenGenes database (version 13.5), which allowed clustering of sequences into operational taxonomic units (OTUs). Alpha-diversity was calculated according to the Simpson index which accounts for both abundance and evenness of the species present. The sequence data have been submitted to the EMBL-EBI database under accession number PRJEB30123.

2.3. Current and efficiency calculations

Current generation was calculated by using the I = E/R equation, while power output of the cells by using the P = I/E equation, where I (A) is the current, E (V) the voltage, R (Ω) the external resistance and P (W) the power. Coulombic efficiency (CE), defined as the fraction of electrons recovered as current versus that in the starting organic matter, was calculated as in the following reported [8]:

$$E = Cp/CTi \times 100\%$$

where Cp is the total Coulombs calculated by integrating the current over time, *C*Ti is the theoretical amount of coulombs that can be produced from either sodium acetate ($i = a$) calculated as:

$$CTi = FbiSiv/Mi$$

where F is Faraday's constant (96 485 C mol^{-1} electrons), bi the number of moles of electrons produced per mole of substrate (b acetate = 8), Si the substrate concentration, and Mi the molecular weight of the substrate (M acetate = 82 g Mol^{-1}) and v is the volume of the reactor. The calculation of power density (mW m^{-2}) and current density (mAm^{-2}) were based on the surface area of one side of the cathode that was of 7 cm^2.

3. Results and discussion

3.1. Electrical performance

The voltage generated during MFC performance was recorded continuously from the inoculation of the bioreactor until the end of the experiment (11 days). A total of 7 voltage cycles were recorded (Fig. 1), with a maximum peak voltage of 0.6 V during the 5th cycle; the voltage values obtained were in line with other work previously reported in the literature [4,35]. To evaluate the MFC performance, i.e. current and power densities of the bioreactor, both polarization and power density curves were acquired (Fig.2). In particular, results reported in Fig.2 show the curves obtained by changing the external resistance (0–10 k Ω) every 30 min and measuring the cell voltage for each different resistance during the stable peak voltage phase. The power curve showed a maximum power density of 565 mW m^{-2} at 500Ω with a corresponding current density of 1.27 Am^{-2}. Also, in this case the values obtained were in line with the results reported in the literature when a similar MFC architecture and feeding substrate were used [35]. The polarization curve could be divided into three regions [8,22,23], corresponding to three different energy losses: activation, ohmic and mass transfer losses. Both bacterial metabolism and electron transfer from the microbial cell membrane to the electrode, affected activation losses. In this case, the polarization curve (Fig. 2) showed a small decrease in the activation losses region compared with the other zones: this result was probably due to an efficient electron transfer from the microbial biofilm to the anode [4,22,23]. Coulombic efficiency (CE), i.e. the electrons recovered as current vs. those present in the starting organic matter, obtained during the test was of 27.6 ± 1.8%, on average, with a maximum of 32% for the period within the second and the last

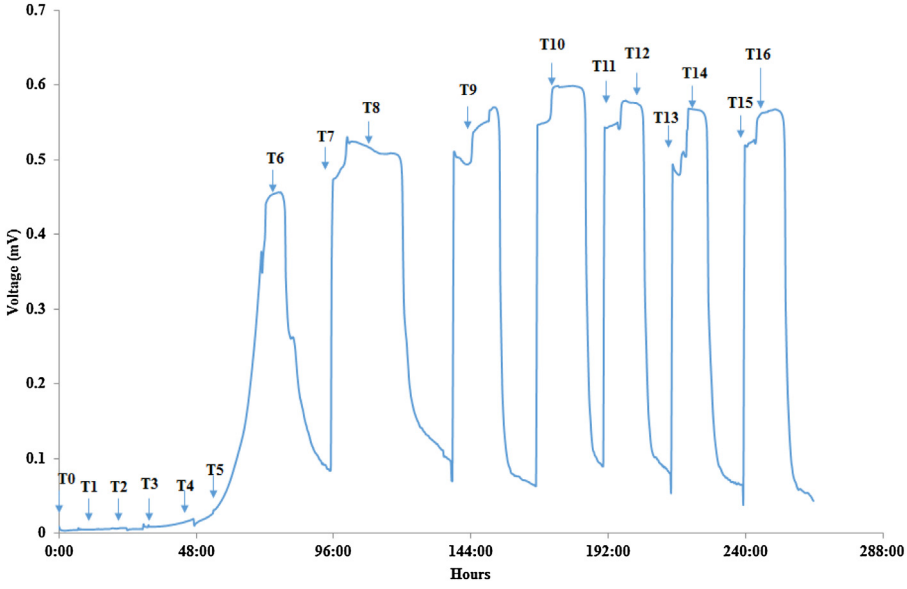

Fig. 1. Voltage cycles obtained during MFC test.

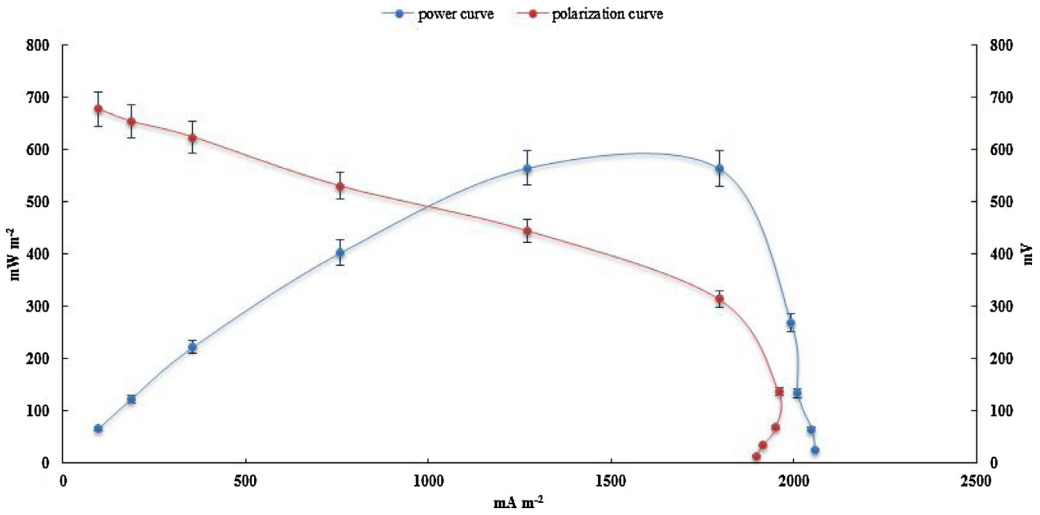

Fig. 2. Polarization and power density curves acquired during MFC test.

voltage cycle. These values were in line with previous data reported in the literature [35,42].

3.2. Microbial population analysis

During the voltage cycles, planktonic and anode biofilm microbial populations were analysed by flow cytometry and by a metataxonomic approach. As indicated in Fig. 1, sixteen samples were taken from planktonic and anode biofilm to analyze the evolution of microbial populations during the entire test length.

3.2.1. Planktonic microbial groups dynamics by flow cytometry

The analyses of planktonic microbial groups by flow cytometry were conducted by labelling separately the microbial cells with SYBR Green I and $DiOC_6$. The flow cytometry plots of all the samples are shown in Fig.S1. Data obtained using SYBR Green I, revealed a simplification of the microbial diversity during the test, in terms of FSC and nucleic acids content (Fig. 3). In fact, the microbial system evolved from at least four groups detected at the beginning of the test (T0, Fig. 3), to two main groups (G1 and G2) during the evolution of the process. These two groups coexisted only at the 11th day, whereas before and after this point, only one of the two was dominant. G1 and G2 groups were similar in terms of the FSC parameter, which was related to the cell dimensions, but they were different in terms of DNA content. G1 was identified as a low DNA content group, while a higher DNA content characterized G2 based on SYBR Green I fluorescence. On the other hand, flow cytometry analysis, based on the

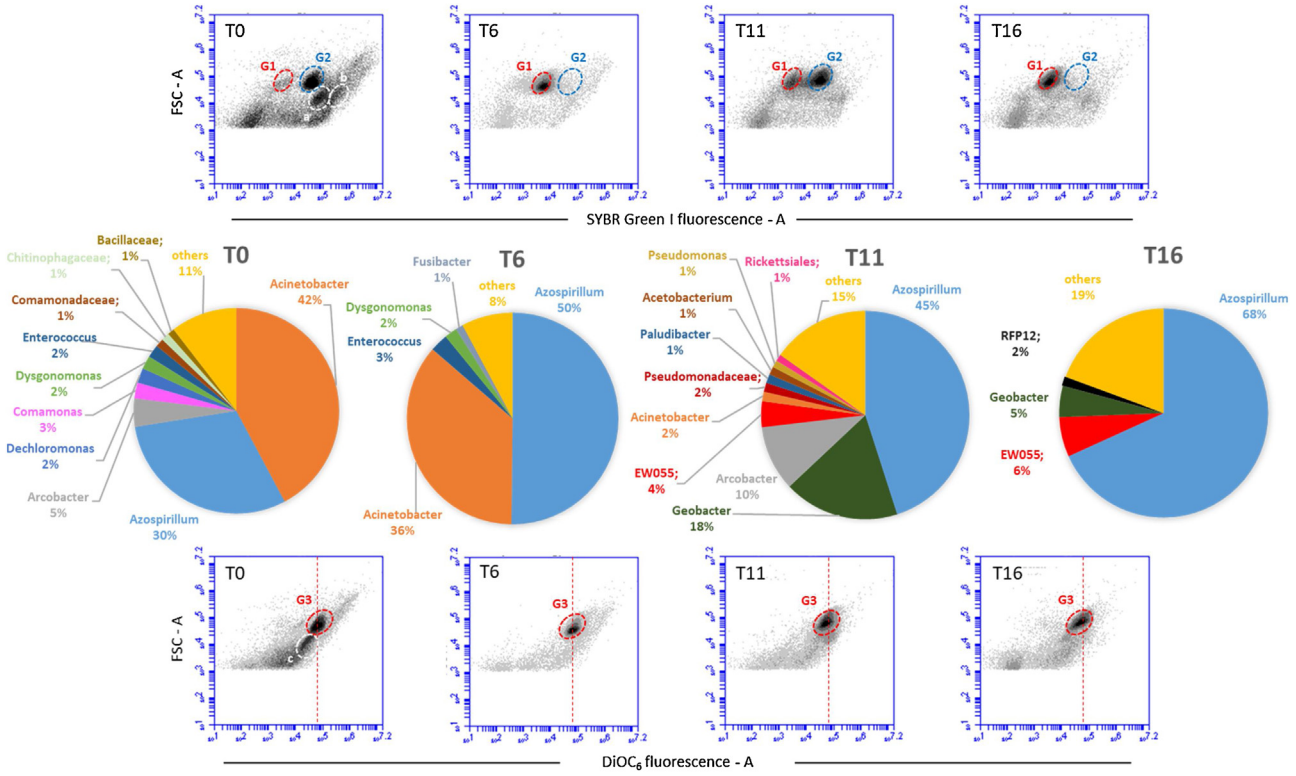

Fig. 3. Comparison between flow cytometry and microbial community structure analysis of MFCs planktonic population.

membrane-potential-sensitive $DiOC_6$ probe, revealed the presence of only one metabolically active group during the electrogeneration process (Fig. 3).

3.2.2. Microbial community structure of the planktonic microbial group dynamics

Being aware of the fact that these cytometric homogeneous populations could be taxonomically heterogeneous and constituted by several microbial species, we were not surprised by the *16S rRNA* gene profiling data obtained, which described a more complex picture even if only few genera were dominating the microbial community. *16S rRNA* gene profiling showed a quite stable composition of the main genera dominating the microbial community concomitantly with a dynamic change in bacterial genera showing lower relative abundances. The alpha diversity was relatively similar in all samples (Fig. S2). At the beginning of the test (T0), the relative genera abundance was of 42% for *Acinetobacter* (commonly found in MFC and MEC biofilms; [24,43]); and of 30% for *Azospirillum* (a known EAB genus; [24,32]), followed by 5% of *Arcobacter,* (a previously reported electroactive bacterial (EAB) genus; [32,44–46]) and other minor genera. Another two genera commonly found in the planktonic and biofilm MFC communities consisted of 3% of *Comamonas* and 2% of *Dysgonomonas* ([45]; Fernando et al., 2014; Sotres et al., 2015). After 3 days (T6; Fig. 3), the data showed an increase in the presence of *Azospirillum* (50%) and a small decrease in *Acinetobacter* (36%). *Enterococcus* [47] was quite stable, 2% at T0 to 3% at T6; *Dysgonomonas* remained at 2% (Fig. 3). Interestingly at T6, when the first significant increase of voltage was measured (Fig. 1), the genus *Geobacter*, one of the most studied EAB genera (Lovely et al., 2010; [13]) was detected even if at low relative abundance (below 1%). During the 5th voltage cycle (T11, Fig. 3) the genera known to be involved in electrical current generation in the MFC process (Beercroft et al., 2015) such as *Geobacter*, *Arcobacter* and *Pseudomonas*, increased their relative abundance compared to T0 and T6 (*Geobacter* 18%, *Arcobacter* 5.9%, *Pseudomonas* 2.6%). At T11 *Azospirillum* remained the dominant genera at 45% of relative abundance. At the end of the test (T16), *Azospirillum,* a genus commonly found in the MFC microbial population [24,32], was still the dominant taxon with a relative abundance of 68%, followed by the order EW055 (6%), the genus *Geobacter* (5%), and the family RFP12. It is interesting to note that *Azospirillum* and *Acinetobacter* were the dominant genera at T0 and T6 while at T11 and T16 *Acinetobacter* decreased below the detectable threshold, whereas the bacterial genera with a relative abundance below 1% (other, Fig. 3) showed together a relative abundance of 15% and 19%, respectively at T11 and T16 (Fig. 3). The comparison between flow cytometry and metataxonomic data led us to hypothesized that the cytometric populations G1 (FSC *vs* SYBR Green I fluorescence) and G3 (FSC *vs* $DIOC_6$ fluorescence) were mainly represented by the *Azospirillum* genus.

3.2.3. Anodic microbial groups dynamics by flow cytometry

Analysis of anodic microbial groups by flow cytometry revealed a more complex picture compared to that obtained for the planktonic groups. Biofilm formation on the anode surface probably interfered with the flow cytometry analysis due to the difficulty of mechanically disrupting the biofilm and releasing free cells in suspension. For these reasons, it was not possible to label microbial groups with the cell-permeating SYBR-Green I. Flow cytometry analysis carried out labelling the microbial groups with the membrane-potential-sensitive $DiOC_6$ probe (Fig. 4) revealed the presence of at least two main groups at T11 and T16. Group G2a, probably composed of two or more not well resolved populations, was present in both samples whereas G1a and G3a were present at T11 and T16 respectively (Fig. 4).

3.2.4. Microbial community structure of the anodic microbial groups dynamics

Microbial community structure analysis of the anode biofilm revealed that in both samples *Geobacter* was the dominant genus with a relative abundance of 28% and 20% at T11 and T16,

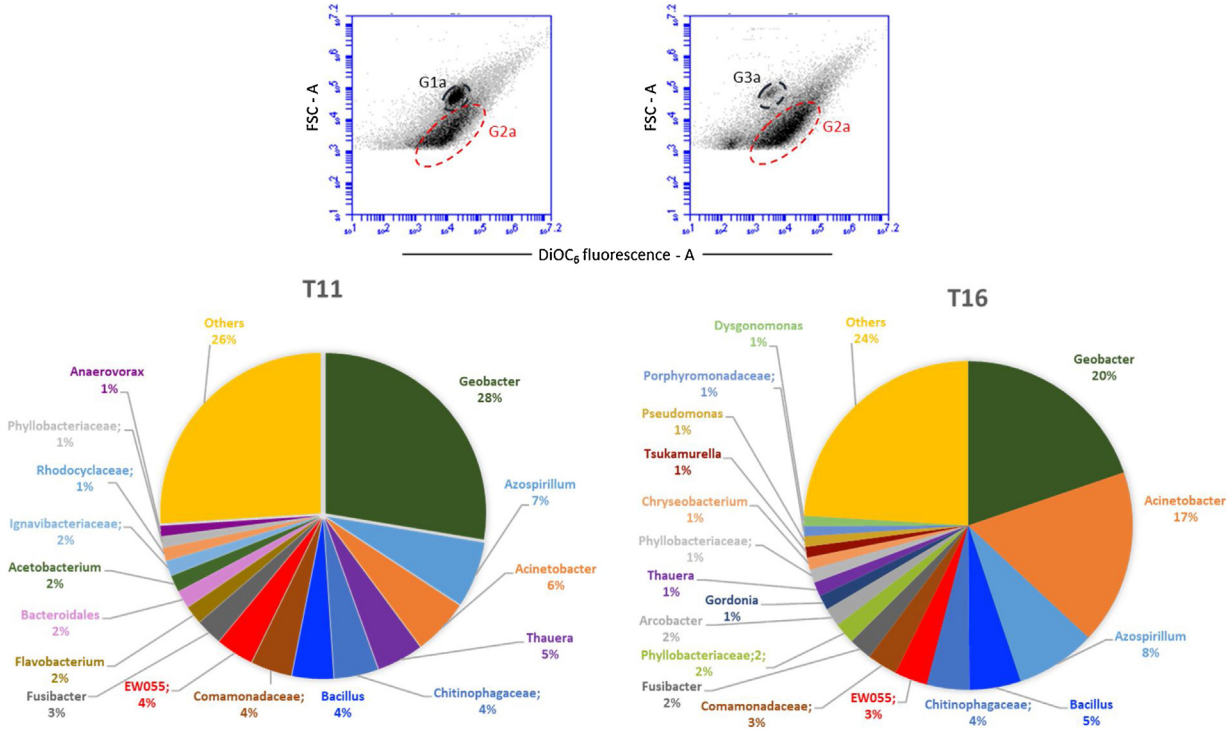

Fig. 4. Comparison between flow cytometry and microbial community structure analysis of MFCs anode biofilm population.

respectively. The alpha diversity was slightly higher in T16 sample compared to T11 sample (Fig. S2). The presence of *Geobacter* in the anode biofilm has been reported frequently in the literature [13,24,48] and it can confirm that the proper acclimation occurred during the test. Interestingly, microbial populations with abundance lower than 1% represented a total of 26% and 24% of the overall microbial community on the anode biofilm (Fig. 4), thus highlighting a high degree of diversity in the anodic microbial biofilm. *Acinetobacter* and *Azospirillum* detected at T11 with a relative abundance respectively of 6% and 7% increased at T16 respectively to 8% and 17% (Fig. 4), which led us to hypothesize an active role for these populations in the electro-generation process. This role was confirmed by other studies where the genus *Acinetobacter* was found within the bacterial communities of different bio-electrochemical systems. In particular, the genus *Acinetobacter*, was found by Choo et al., [49] as the predominant γ-proteobacteria (33.6%) within the bacterial communities in a mediator-less MFC fed with glucose and glutamate [49]. Han et al., 2011 reported the presence of *Acinetobacter junii NIU-Y8* in an anode bacterial communities of a single chamber MFC fed with activated sludge for wine-containing wastewater treatment. On the other hand, [50] reported the presence of *Acinetobacter calcoaceticus* within the biocathode communities able to utilize the cathode electrode as the electron donor to perform oxygen reduction reaction (ORR). These studies confirmed that the genus *Acinetobacter*, is involved not only during the extracellular electrons transfer to an anode but also during the bio electro-catalytic reactions occurred over the cathode. 3.2.3 Coulombic Efficiency vs microbial population composition

The comparison between the Coulombic Efficiency (CE) obtained during the voltage cycles and the amount of *Geobacter, Acinetobacter* and *Azospirillum* genera on the planktonic and the anode biofilm were compared (Fig. 5). In particular, the higher CE value (32%) was obtained during T16. As reported in the description of the microbial population, the T16 matches with the higher abundance (68%) of *Azospirillum* taxon in the planktonic phase while, in the anode biofilm, the genus *Acinetobacter* reached 17% of abundance, and *Geobacter* decreased from 28% at T11 to 20% at T16. Passing from T11 to T16, CE value increased (from 29% to 32%, Fig. 5), simultaneously with the increase of the genus *Acinetobacter*, indicating the potential role of the genus *Acinetobacter* in the electro-generation process [24,32,43]. Unfortunately, due to the little number of samples obtained, was not possible to confirm this trend by a statistical analysis. Further investigation of the *Acinetobacter* genus should be conducted to better understand the capability of this genus in extracellular electron transport. These results revealed how using these high-throughput screening techniques, it was possible to investigate in depth the microbial populations' evolution and dynamic both in the planktonic phase and within the anode electrode biofilm of a microbial fuel cell reactor. The implication of dynamic and changes of microbial communities reported during the start-up phase (Figs. 3 and S1) led to a slow down to the achieving of the stable voltage generation phase. In this way, FC could be a useful tool for monitoring the dynamic of the microbial population in the early start-up phase and it could be used to predict if, the FC dynamic changes showed, are in line with respect to the data reported in this work or in literature. According with the literature an improper start -up phase could lead to decrease of voltage generation during the stable phase due to the increase of the overpotential [4,50]. Several studies demonstrated the possibility to reduce the start-up time by using several techniques. One of the most efficient methods for starting up a new MFC reactor is to use the effluent coming from an existing reactor treating the same type of substrate [51,3]. Another technique is, for example, adding specific electron acceptor as Fe (III) or Fumarate to promote the growth of known exoelectrogenic

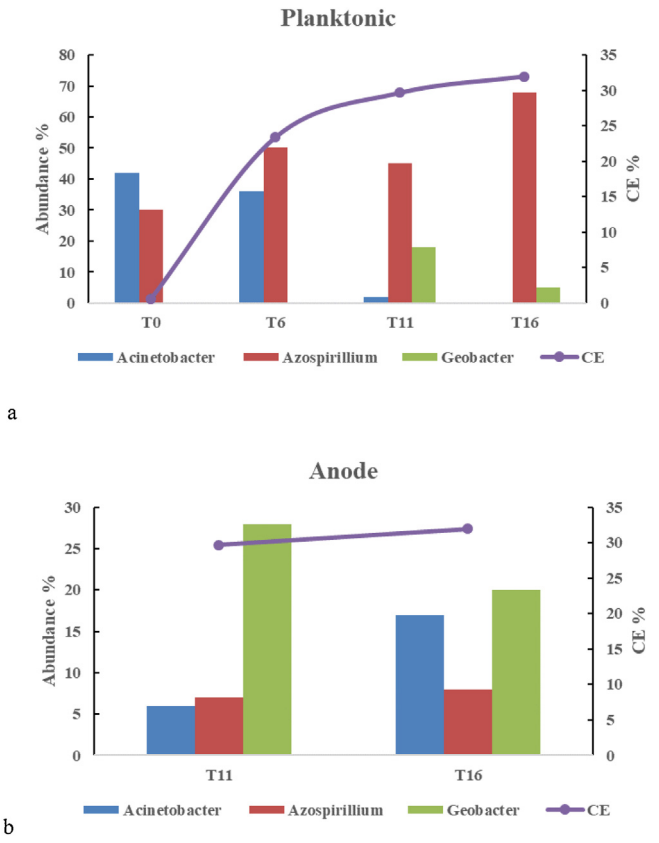

Fig. 5. Comparison between the Coulombic Efficiency (CE) obtained during the voltage cycles and the amount of *Geobacter, Acinetobacter* and *Azospirillum* genera on the planktonic (a) and the anode biofilm (b).

bacteria [52] or increase the conductivity of the wastewater used. In fact, a low conductivity can limit current densities, and thus it may allow other non-exoelectrogenic bacteria to colonize the electrodes and inhibit growth of exoelectrogenic bacteria [51]. From this point, these techniques can, in the future, improve significantly our understanding of the microbial community; moreover, they can be used as a tool to predict "on line" the changes in bacterial community composition not only in microbial electrochemical technologies but also in all microbial applied technologies.

4. Conclusions

The overall data obtained, clearly indicated that multi-parameter flow cytometry combined with the Illumina sequencing are useful tools for studying the changes in microbial communities during the start-up and the following stable voltage generation of an MFC bioreactor. In particular, the presence of *Azospirillum* and *Acinetobacter* as mains genera found within the bacterial communities suggest using these two genera as quality probe for understanding the correct acclimation of the MFC during the start-up phase. Furthermore, it has been shown that the monitoring of planktonic population, rather than the less accessible anode biofilm, was in good agreement with the MFC voltage generation/evolution.

Conflict of interest

This manuscript describes original work and is not under consideration by any other journal. All authors approved the manuscript and this submission.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00310.

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