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Enrichment of *Clostridia* Enhances *Geobacter* population and Electron Harvesting in a Complex Electroactive Biofilm

Caroline Rivalland[a], Fatima Radouani[a], Silvina Gonzalez-Rizzo[b], Florent Robert[a], Paule Salvin[a,*]

[a]Laboratoire des Matériaux et Molécule en Milieu Agressif L3MA EA7526, UFR STE, Université des Antilles, Schœlcher, France
[b]Institut de Systématique, Evolution, Biodiversité (ISYEB), Muséum national d'Histoire Naturelle, CNRS, Sorbonne Université, EPHE, Université des Antilles, Pointe-à-Pitre, France
[*]Corresponding author

Email address of the corresponding author: paule.salvin@univ-antilles.fr

Abstract

Current research on microbial fuel cell or microbial electrolysis cell dealt with finding new electroactive bacteria and understanding the mechanisms of electronic exchange. Complex consortia allowed to obtain better performances than pure cultures in part thanks to inter-species cooperation. However, the role of each bacterium in a complex biofilm in the electron harvest on an electrode remains unclear. Thus, we combined electrochemical monitoring of electron exchange and high throughput sequencing analysis in order to describe the bacterial composition and the electroactive performance of mangrove mud biofilms. In this study, secondary electroactive biofilms were formed on carbon electrodes from *Desulfuromonas*-dominated inoculum of pre-formed bioanodes. The performances and the *Desulfuromonas*-dominated profile were the same as those of primary bioanodes when the planktonic community was conserved. However, a *Clostridium* enrichment allowed to restore the performance in maximal current densities promoting an increase of *Geobacter* population, becoming the most dominant group among the *Deltaproteobacteria*, replacing *Desulfuromonas*. These results highlight a positive collaboration between *Clostridium* and *Geobacter* spp. helping a bacterial population to achieve with the depletion of their environment. Our study provides new insight into relationships between dominant electroactive bacteria and other bacteria species living in an organic matter-rich environment as mangrove sediments.

Keywords

Electroactive biofilm, Bioanodes, *Clostridium*, *Geobacter*, *Desulfuromonas*, Bacterial interaction, Mangrove sediments

1. Introduction

Electroactive (EA) bacteria find their interest in being catalysts of redox reactions in various bio-electrochemical systems (BES) such as the microbial fuel cell (MFC for generation of electricity) or the microbial electrolytic cell (MEC for production of molecules of interest such as hydrogen). Members of Proteobacteria and Firmicutes were identified as dominant bacteria in electroactive biofilms (EAB), particularly in those formed on anode within acetate supplement media [1-3]. Among Proteobacteria, the Geobacteraceae (e.g. *Geobacter sulfurreducens*, *G. metallireducens,* or *G. Lovleyi)*, is one of the best known and described families concerning their EA properties [4-7]. *Desulfuromonas acetexigens* is also considered as EA bacterium. Biofilms of pure culture of this bacterium were capable to produce considerable current density (> 9 A/m^2) in a short period of potential-induced growth using acetate as an electron donor [8]. Members of Firmicutes have been described as less electrochemically active than Proteobacteria. Nevertheless, *Clostridium butyricum* and *Clostridium beijerinckii* were referenced as isolated and inoculated bacteria in MFC successfully even if the currents generated were of low intensity [9,10].

Studies show that the complex communities directly retrieve from natural environments (sediments, marine biofilms ...) or anthropogenic sources (industrial or domestic wastewater, compost ...) are more effective in BES [1]. This effectiveness can be explained by cooperative interactions between exoelectrogenics bacteria and other bacteria species. In fact, non-EA bacteria can enhance the current generation by providing a suitable environment for EA bacteria, such as maintaining anaerobic conditions by removing oxygen [11,12]. Another type of interactions involves easing access to substrate by hydrolyzing organic matter to a simple substrate useful for EA bacteria [13,14]. Positive interactions between bacteria also exist at the electron exchange level by direct cell-cell communication, or indirect electron transfer via pili or conductive microbial appendages [15].
Among the natural environments, mangrove sediments have already been identified as efficient EA inoculum in MEC and MFC [5,16-19]. EA biofilms obtained from bacterial community of mangrove sediments have either *Geobacter* or *Desulfuromonas* dominance depending on NaCl concentration [20].
Moreover, previous work has shown a decrease of the bacterial diversity in EA biofilms from mangrove sediments through the formation of successive generations of biofilms [21]. An unexplained drop in current intensity over generations was recorded despite the recurrence of an EA biofilm community dominated by *Desulfuromonas.* Indeed, the decrease of currents was accompanied by a decline of *Clostridia* class members in the biofilms, suggesting a potential link between Clostridia and yields of currents over generations of biofilms. Thus, we compared the bacterial composition of secondary biofilms under different electrolyte conditions (depleted electrolyte and after Clostridia enrichment) to highlight the role of Clostridia in the electroactive biofilms obtained from mangrove sediments. Clostridia enrichment of electrolytes aimed to increase its population in EA biofilms to allow an equal or better electrochemical performance over the next generation of secondary biofilms formation. In addition, we present electrochemical profiles of each multigenerational biofilm generated to correlate them with the microbial diversity obtained by high-throughput sequencing (Miseq).

2. Materials and Methods

2.1. Electroactive Biofilms Formation

Experiments were done in 25°C air-conditioned room. To favor biofilm growth, sodium acetate was provided as a carbon source, as it is a simple molecule easy to degrade by the respiratory bacteria. Acetate was provided at initial time and at several times of the experiment specifically after a peak of electrical activity. The substrate concentration change over a batch cycle was measured with HPLC (Dionex UltiMate 3000 - Thermo Scientific, Rezex ROA-Organic Acid H+ (8%) column – Phenomenex).

2.1.1. Primary Biofilms

Primary electroactive biofilms (named PB throughout the text) were formed during 2.5 months by immersing poised potential carbon electrodes (WE: working electrode, 16cm^2 carbon tissue, PaxiTech) in five glass reactors. Each reactor was filled with 500mL of mangrove sediments mixed with 2 L of distilled water according to methodology described previously [21]. The mix sediments + water had a conductivity of 10 ±2 mS/cm. The potential of -0.2 V versus saturated calomel electrode (SCE: reference electrode, Bio-Logic) was imposed on each WE via a multi-channel potentiostat (VMP3, BioLogic® SA). A grid platinum served as the counter-electrode and was placed close to the WE and the SCE. For the primary biofilms, a final substrate concentration of 10 mM was chosen for the first additions (complete peaks), and then 20 mM for the others following.

2.1.2. Secondary Biofilms

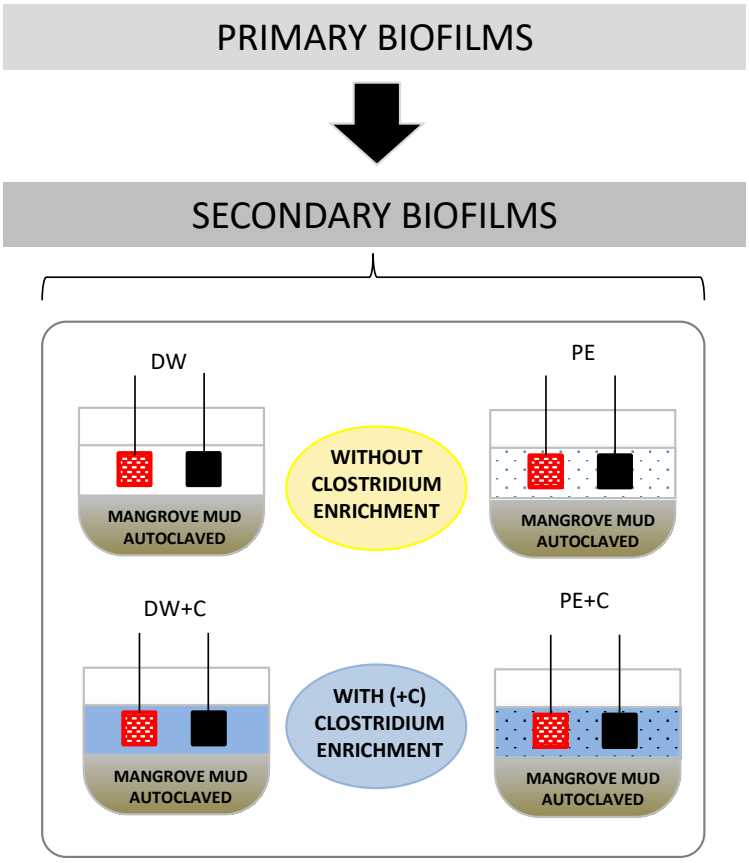

Fig.1. Secondary electroactive biofilms' (SB) formation. The SBs were formed on a clean carbon electrode (black square) from a primary biofilm (red square) extracted from a previous reactor under 2 different conditions of electrolyte: Distilled water (DW, blank) and Primary biofilm electrolyte (PE, dotted). For both conditions, a supplemental reactor was enriched by *Clostridium* spp. (+C in blue).

Secondary electroactive biofilms (SBs) were formed under -0.2 V/SCE on clean carbon WEs. These biofilms were obtained from primary biofilms (PBs) still fixed on their carbon electrode and placing them as inoculum, next to new clean electrodes (Fig.1). The electrolytes of SB reactors were composed of 500 mL of sterile (autoclave) mangrove mud (MM) plus 2 L of a liquid phase which were either liquid electrolyte from a primary reactor (PE condition) or distilled water (DW condition). Under PE condition, the electrolyte had a conductivity of 20 ±2 mS/cm. A third condition was 0.47 µm then 0.2 µm filtrated PB's liquid electrolyte (FPE condition). In function of the conditions, the electroactive biofilms obtained on secondary electrodes were called through the article as follow: SB_PE, SB_DW, SB_FPE. Each experiment was made in duplicate and numbered with 1 and 2. Acetate was brought in the electrolyte at the initial time with a concentration of 20 mM and then was adjusted at the same concentration at different other times of the experiment.

2.1.3. Clostridium Enrichment

Enrichment of *Clostridium* spp. was performed incubating mangrove sediments (MM) in Reinforced Clostridial Medium (RCM) in a thermostatically controlled chamber at 30°C. Degassing was done regularly all along the enrichment that took place on a period of one to two weeks. Distribution of major phyla in MM inoculum before and after enrichment was looked at (Fig.S1a) as well as the distribution of bacterial genera among Firmicutes after enrichment (Fig.S1b). 50 mL of Clostridia-enriched mangrove sediments were added in the reactors at the initial time. The electroactive biofilms formed in contact with the enrichment were called in the text of this article: SB_PE+C (for PE condition) or SB_DW+C (for DW condition) following the nature of the electrolyte used.

2.2. Electrochemical measurements

The electrical activity of the biofilms was followed recording the intensity I of the current passing between WE and counter electrode versus time t. The measurement and the collect of data were realized via Ec-Lab software (version 10.12, BioLogic® SA). Chronoamperogramms were drawn showing density of current J versus time. J values were obtained dividing I by the projected surface of the WE and were expressed in A/m^2.

Electroactive performance of electroactive biofilms was evaluated based on the maximal current density achieved during the formation procedure. PBs, obtained in the same conditions, gave reproducible measurements with an average value of 9.1 ± 0.3 A/m^2 (triplicates) and maximal current densities tended to a plate between 7 and 10 A/m^2 (Fig.S2) before being harvested to serve as inoculum in SB's reactor.

For SBs, the three first peaks recorded after the supply of acetate at initial time and the two adjustments after were considered for the electroactivity evaluation. Duplicates 1 and 2 in each condition of electrolyte have shown similar electrochemical profiles. Thus, the mean of maximal current densities was presented as results and only one graph by pair was represented in this article for simplification.

Electrochemical data analysis allowed the calculation of coulombic efficiency C_E which is the percentage of electrons efficiently harvested through bacterial transfer to the working electrode. C_E is calculated according to (1).

$C_E = M_S.\int I.dt/(F.b_{es}.v_{an}.\Delta C)$ (1)

$\int I.dt$ represents the integration of current over time; M_S is the molecular weight of the substrate; ΔC is the substrate concentration change over a batch cycle; F is Faraday's constant; b_{es} is the number of moles of electrons released with the complete oxidation of one mole of the substrate; v_{an} is the volume of electrolyte in the anode compartment.

2.3. Bacterial community analysis

2.3.1. DNA preparation and sequencing

Bacterial DNA was extracted from biofilms and inocula samples using the FastDNA™ SPIN Kit for Soil and the FastPrep® instrument (MP Biomedicals, Santa Ana, CA) according to manufacturer's instructions.
V3 region of 16S rRNA gene was amplified using the forward e334f 5'-CCAGACTCCTACGGGAGGCAGC-3' and the reverse e534r 5'-ATTACCGCGGCTGCTGGC-3' primers. PCRs were run in 50 µL reaction mixture containing 10 ng of extracted DNA, 1X of PCR buffer, 0.5 µM of each primer, 0.2 mM of dNTPs and 4 units of the Taq polymerase (MolTaq 16S DNA polymerase, MEDIANE or MTP Taq DNA polymerase, SIGMA).
PCR amplifications were performed as follows: 95°C for 10 min, 40 cycles of 94°C 30s, 64°C 30s, 72°C 30s and finally 72°C for 10 min. Amplified fragments of 202 bp were checked in 1% GelRed agarose gel and sequenced by France Genomique® platform using an Illumina ® MiSeq®.
High-throughput sequencing data were loaded into SILVA ngs® database in order to study the bacterial composition of electroactive biofilms. Genus relative abundances were determined with Krona® charts (Hierarchical Data Browser). MiSeq sequences were also processed with Mothur and Excel software to precise the species that were the most abundant in each sample. For this, the most abundant sequence was determined and then aligned with GenBank database using BLASTN (NCBI).

2.3.2. High-throughput data statistical analysis

Pearson's correlation coefficient r was used to measure the strength of the association between variables [22]. Here, variables were the relative abundances regarding the different species of interest that were highlighted during the study. Positive correlation indicates that both variables increase or decrease together, whereas negative correlation indicates that solely one variable increases, or decreases, and vice versa. The formula for two datasets {x1,..., xn} and {y1,..., yn} both containing n values is:

$$r=[n.\sum(xy)-\sum x.\sum y]/((n.\sum x^2-(\sum x)^2)^{1/2}.(n.\sum y^2-(\sum y)^2)^{1/2}) \quad (2)$$

Mothur software was chosen to analyse high-throughput data. MiSeq data statistical pre-processing and analysis were carried out following MiSeq standardized operating procedure [23].

3. Results

3.1. Electrochemical performance of biofilms

Chronoamperometry has proven its worth in detecting the electroactivity of bacterial consortia from complex inoculum source. This electrochemical method consists in imposing a stable redox potential on an electrode, immersed in an electrolyte containing the inoculum. Thus, the electrode constitutes an attractive site for EA bacteria which, adhere to the material surface, then use it as an acceptor or electron donor depending on the redox potential [24-26]. However, this method has its limits because non-EA bacteria may be selected and be present in the complex EA biofilm, limiting the performance of the bioelectrode in harvesting electrons from the medium. To solve this problem, several research teams formed EA biofilms enriched in EA bacteria thanks to the multigenerational formation of electroactive biofilms [21, 27-31]. Secondary or even tertiary EA biofilms were obtained by resuspension of a primary bacterial EA biofilm obtained from a complex inoculum (MFC anode, wastewater, mangrove sediments, etc.). Re-inoculation with the primary biofilm reduces the presence of electrochemically inactive bacteria and, in general, is accompanied by an increase in electrical performance in the case of MFCs.

Table 1. Maximal current density in A/m^2 obtained during primary electroactive biofilms' (PBs) and secondary electroactive biofilms' (SBs) formation on -0.2 V/SCE poised carbon cloth immersed under different conditions of electrolyte (1 and 2 were duplicate, realized under the same electrolyte conditions).

Initial Electrolyte condition	Biofilm Name	J_{max} in A/m^2	**Average J_{max} with standard deviation** in A/m^2
Mangrove mud (MM) + Distilled water (DW)	PB1 PB2 PB3	9.0 8.8 9.4	9.1 ±0.3
Sterile MM + Primary reactor electrolyte (PE)	SB1_PE SB2_PE	8.4 9.2	8.8 ±0.4
Sterile MM + Filtrated PE	SB3_FPE	5.0	
Sterile MM + DW	SB1_DW SB2_DW	6.6 4.0	5.3 ±1.3
Sterile MM + PE + Clostridium enrichment (C)	SB1_PE+C SB2_PE+C	9.3 6.5	7.9 ±1.4
Sterile MM + DW + C	SB1_DW+C SB2_DW+C	8.8 8.0	8.4 ± 0.4

In this study, secondary electroactive biofilms (SBs) were formed on clean electrodes using pre-formed mangrove sediment EABs as inocula (PBs). Maximal current densities J_{max} were recorded during SBs' growth under different electrolyte conditions. With or without *Clostridium* enrichment, the reuse of a primary reactor's electrolyte (PE & PE+C conditions) led to J_{max} performance similar to those observed in PBs, with an average value of 8.4 ±1.3

A/m^2 for SBs (Table 1). On the other hand, without *Clostridium* enrichment, in DW and FPE conditions, J_{max} were up to two times lower than those of PBs, and their average value was equivalent to 5.2 ±1.3 A/m^2.
This result shows that bacteria of the PBs community alone could not recover the observed effectiveness of PE conditions. Indeed, it seems important to conserve the planktonic bacterial community to reach it. Neither the conductivity nor the chemical composition of the electrolytes could explain the decrease in performance between SB_DW and SB_PE. The experiences with the maintenance of chemical compounds as well as the conductivity of the primary solution (FPE condition) ruled out these possibilities.
Otherwise, the lag period, corresponding to the attachment of the first bacteria on the solid electrodes [27,32,33], was shortened under PE condition while it took several days under DW and FPE conditions (Fig.2). Indeed, curves under PE condition had a strong slope almost immediately (nearly a day) after the start of the experiment while those under DW and FPE conditions showed significant increases in their slope between 5 and 14 days. Unlike under PE condition, the bacterial concentration was low at the start of the experiment and did not allow rapid colonization of the new electrode.

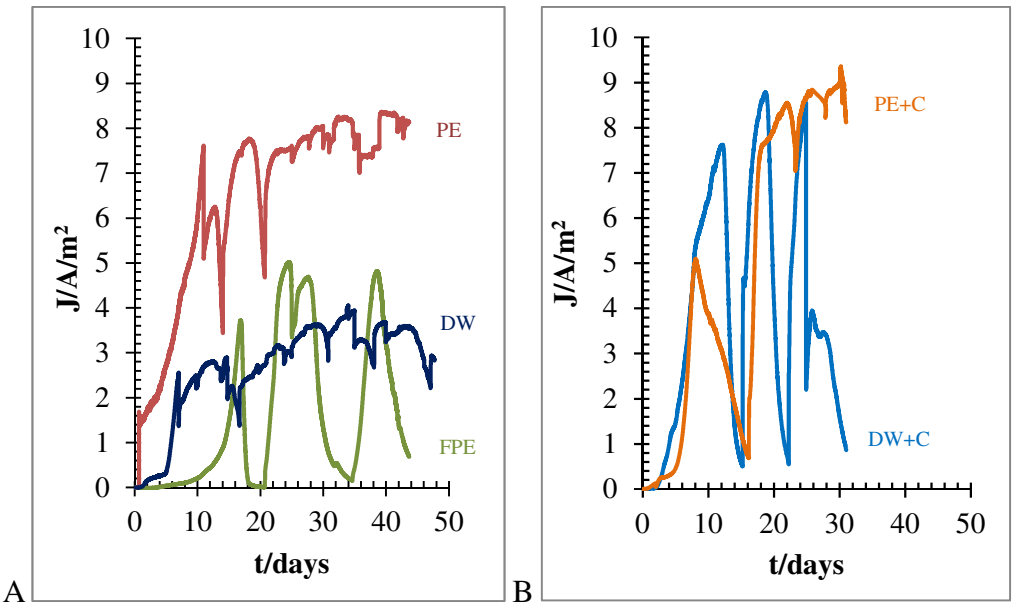

Fig.2. Current densities versus time recorded during formation of SBs under different conditions of electrolyte (PE: PB's electrolyte; DW: distilled water; FPE: filtrated PB's electrolyte) without (A) and with (A) Clostridium enrichment (+C).

Finally, the most important electrochemical result was that the enrichment in *Clostridium* under DW condition allowed to reinforce the ability to harvest electrons of SBs. This result shows a better performance in J_{max} (8.4 ±0.6 A/m^2, equivalent to PBs & SB_PEs'ones) with a shortened latency time of 2 days (Fig.2). This observation seems to support the hypothesis that an ultra-minor bacterial species in the starting inoculum could be partly responsible for the successful electron transfer of the electroactive biofilm. In order to explain this phenomenon, it was relevant to analyze the microbial composition of second generation of biofilms and to identify the putative modifications linked to the *Clostridium* enrichment.

3.2. Biological results

3.2.1 Microbial composition of electroactive biofilms

To study the microbial composition of electroactive biofilms, analysis of 16S rRNA was conducted in duplicate for every electrolyte condition. Amplicon sequencing analysis revealed a large heterogeneity of the relative abundance of bacterial communities at the phylum level among the electrolyte conditions (Fig.3). In SB1_DW electrolyte reactor, the most abundant bacterial phylum was Bacteroidetes (43%) followed by Proteobacteria (19%). In contrast, for SB2_DW, Proteobacteria (37%) was the most abundant phylum followed by Mollicutes (23%) and Bacteroidetes (16%). In primary electrolyte (PE) conserved in second reactors, bacteria belonging to Mollicutes are predominant in both replicates (45% and 41%), followed by the phylum Bacteroidetes with a relative abundance of 24% and 33% for SB1_PE and SB2_PE respectively (Fig.3). Surprisingly, the bacteria belonging to Spirochaetales was noticed solely in unenriched biofilms samples. In the other hand, bacteria belonging to the Proteobacteria were the most abundant phylum (50-79%) in electrolytes biofilms enriched by *Clostridium* spp.
It is widely known that the highest current densities observed to date come from mixed cultures that are usually dominated by Deltaproteobacteria of different genera as *Geobacter* [8].

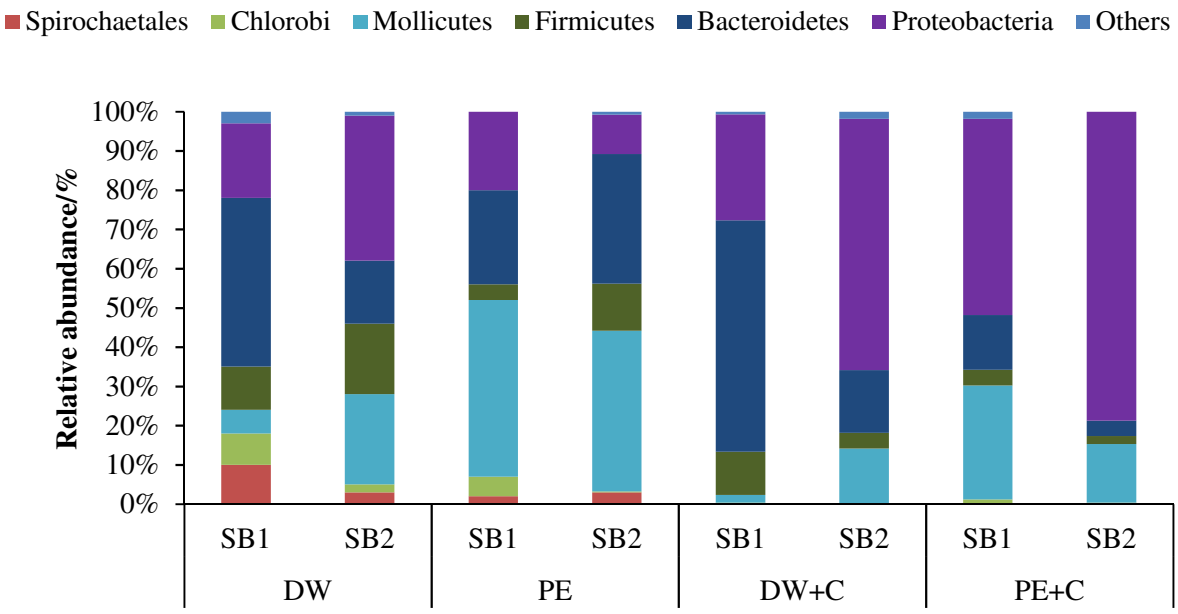

Fig.3. Relative abundance of bacterial groups at the phylum level in Secondary electroactive Biofilms (SB) made in duplicate (1 and 2), with distilled water (DW), primary electrolyte (PE) without or with Clostridium enrichment (+C).

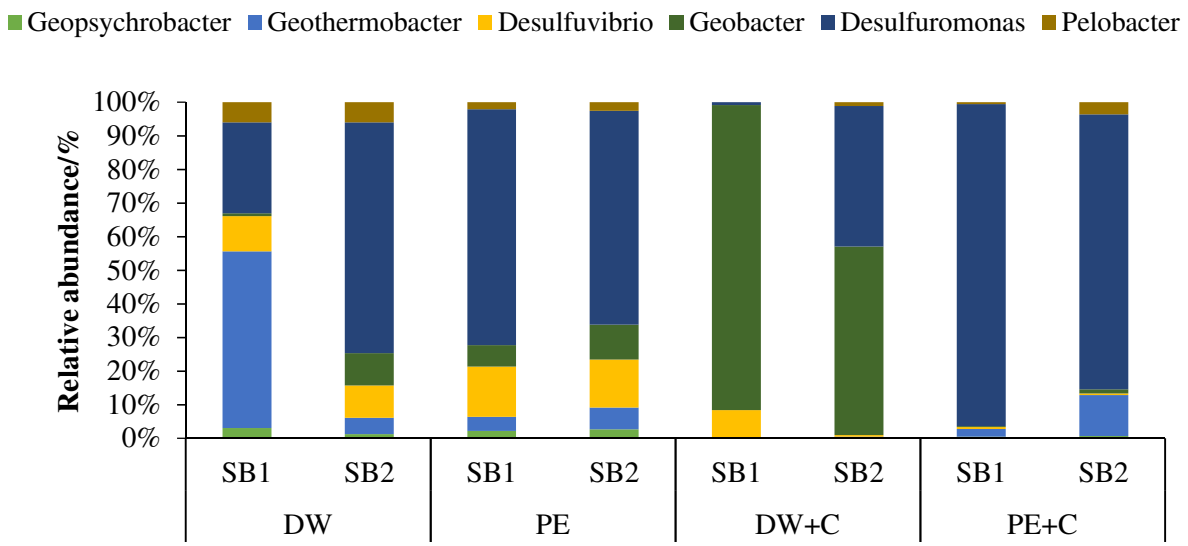

Fig.4. Relative abundance of bacterial community at the genus level belonging to deltaproteobacteria class (proteobacteria) in Secondary electroactive Biofilms (SB) made in duplicate (1 and 2), with distilled water (DW), primary electrolyte (PE) without or with Clostridium enrichment (+C).

Thus, we then analyzed at the genus level the relative abundance of Deltaproteobacteria in our electroactive biofilms (Fig.4). The genus *Desulfuromonas* are dominant (49-84%) in all biofilms except the distilled water electrolyte biofilm enriched by *Clostridium* (DW+C). Interestingly, in the absence of primary electrolyte, SB1_DW+C and SB2_DW+C, an occurrence of *Geobacter* was predominant.

3.2.2. Impact of Clostridium enrichment

In order to determine whether the presence of *Clostridium* enrichment could have an effect on the electrochemical activity of biofilms, an enrichment in *Clostridium* were done taking mangrove mud (MM) as an inoculum and reinforced Clostridial medium (RCM). The enrichment was done in two steps: the first step was to control the enrichment in *Clostridia* and to reduce *taxa* numbers, the second step allowed a reduction of relative abundance in the inoculum. This result showed an enrichment of phylum Firmicutes in MM+RCM, and Clostridia was the most abundant Class (Fig.S1).
To study the impact enrichment produced by *Clostridium*, community of *Clostridium sensu stricto* was investigated (Fig.5). This genus was selected because it was undetectable in the natural inoculum and enriched to approximately 20% of the total bacterial population in the inoculum using RCM medium. Thus, only secondary biofilms with Distilled water as electrolyte (SB1_DW and SB2_DW) were enriched in *Clostridium sensu stricto* in contrast to other conditions. As mentioned before, the relative abondance of *Clostridium sensu stricto* was correlated with the presence of *Geobacter* spp. These results indicate a putative correlation between *Geobacter* and *Clostridium.*

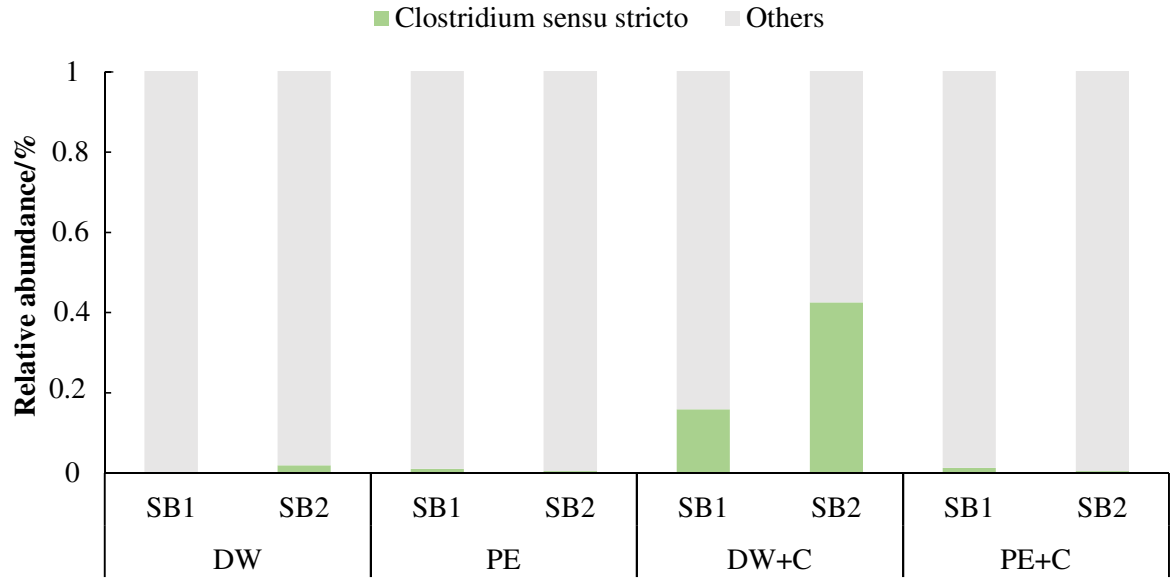

Fig.5. Relative abundance of *Clostridium sensu stricto* among total community in secondary biofilm (SB) made in duplicate (1 and 2) in distilled water (DW) or Primary electrolyte (PE) without and with enrichment of Clostridium (+C). Only the <1 % values are showed on this graph.

4. Discussion

The use of preformed anode biofilms as inoculum allowed the formation of secondary electroactive biofilms. SBs showed different performance depending on the presence or absence of an initial planktonic bacterial community (PBC), and also depending on the composition of this community. The presence of the complex PBC enhanced current densities equivalent to those obtained with the primary reactors. The absence of the PBC at the start of the experiment showed reduced performance suggesting that the effectiveness of current intensity is directly linked to the presence of the planktonic community (Fig.6). Planktonic cells as well as biofilms can perform electronic transfer in BES. However, planktonic species could only use mediators in indirect transfer [34].

A decrease in conductivity by dilution or the lack of essential electronic shuttles (metabolites) for indirect transfers are factors that were ruled out by the experiments performed. Moreover, the addition of a PBC mainly composed of *Clostridium* spp. also restored similar primary electrical performance in secondary reactors. These results suggest that in addition to electroactive bacteria adhered to the electrode, other bacterial species contribute to explain the overall electronic transfer recorded. Altogether, our results suggest that the major electron transfer mechanism apply by biofilms intrinsic species was direct (via cytochromes or pili), while planktonic microorganisms would establish the electroactivity and participate in the indirect electron transfer to the anode.

The microbial composition of biofilms showed a similar diversity of bacteria communities with a concomitant dominance of Proteobacteria, Bacteroidetes, Mollicutes, and Spirochetes despite the difference of the electrolyte conditions used. However, biofilms of *Clostridium* enrichment revealed a quasi-systematic predominance of Proteobacteria and a decrease of the microbial composition diversity in the biofilms. One mechanism by which this might occur is the ability of a population to rapidly colonize new niches as they arise. Individuals of another species would then have fewer unoccupied niches in which to gain a foothold [35].

Proteobacteria typically dominate within biofilms increasing the diversification and competitiveness of a population against other species.
Furthermore, the relative abundance of Deltaproteobacteria on the SBs highlights a dominance of *Desulfuromonas* spp. in primary electrolyte and distilled water conditions despite the difference in electroactive performances of related biofilms. Other studies reported a similar convergence of microbial composition predominant by *Desulfuromonas* spp. in an over 340 h growth matured biofilm [8,36-37]. Katuri et al. described that the bacteria belonging to *Desulfuromonas* are a major contributor to the current generation [8]. However, in our study the abundance of *Desulfuromonas* spp. was not correlated with the effectiveness of the electroactive biofilms. Therefore, it appears that other bacteria species could contribute to the electroactive performance of the studied biofilms.
A previous study on the multigenerational formation of EABs from mangrove sediments correlated decreasing abundance of bacteria species with decreasing of electrochemical performances [21]. High-throughput sequencing data had notably highlighted a drastic loss in the abundance of a specific bacteria belonging to Clostridia class during the formation of secondary and tertiary biofilms suggesting a putative role of these bacteria on currents generation of electroactive biofilms. In our study, the syntrophic role of Clostridia was studied by inoculating the electrolytes of SBs with Clostridia enriched medium. Our results show a significant colonization of *Geobacter* spp. that might result from planktonic Clostridia enrichment in the absence of the initial planktonic community (Fig. 6). Moreover, the quasi-perfect Good Pearson's correlation coefficient between *Geobacter spp* and *Clostridium sensu stricto* (Table 2) confirmed these results, suggesting syntrophic cooperation or cross-feeding interaction between *Geobacter* spp. and hydrogen-oxidizing anaerobic bacteria, such as *Clostridium* described in co-culture conditions [38,39]. The syntrophic relationship observed could be related to the establishment of lower redox potentials, potentially caused by the presence of Clostridia [40,41]. *Geobacter* spp., often described as strictly anaerobic [42], require the medium to be kept at a very low (negative) potential during growth [43].

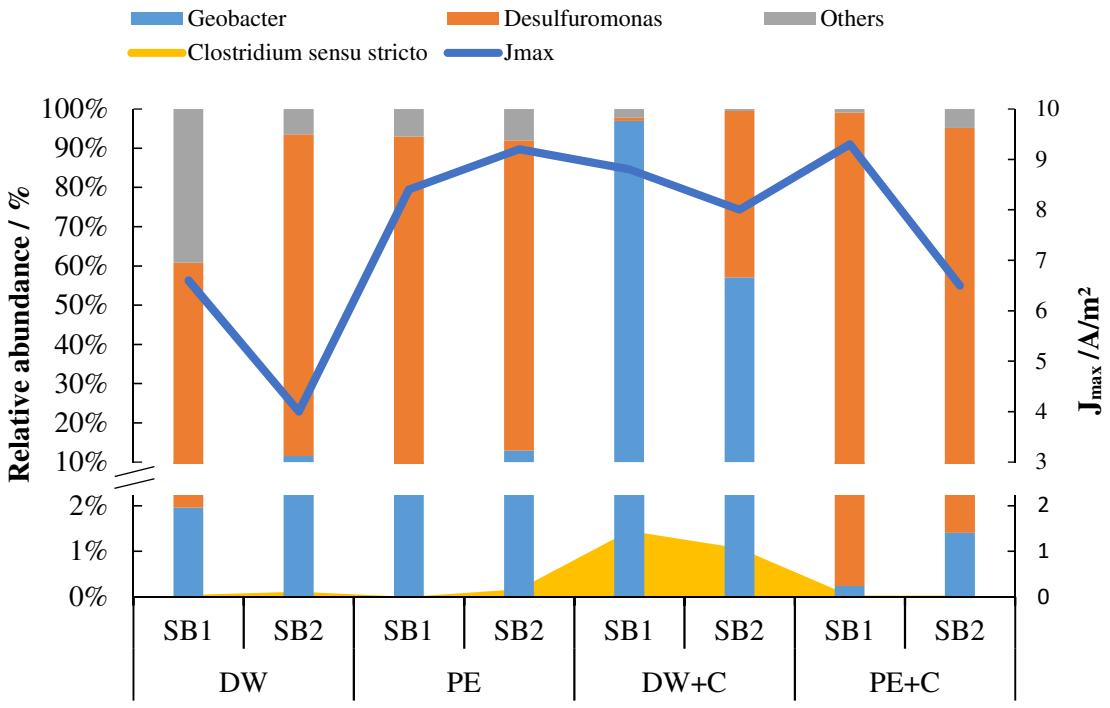

Fig.6. Correlation between the relative abundance of *Geobacter* spp. (blue bars) and *Desulfuromonas* spp. (orange bars) and the maximal electrochemical performance recorded (blue line) related to the proportion of *Clostridium sensu stricto* present (yellow area) in the secondary electroactive biofilms (DW: distilled water as electrolyte, PE: primary reactor supernatant as electrolyte, +C: with Clostridium enrichment) made in duplicate (1 & 2).

Thus, the increase in *Geobacter* spp. relative abundance would be subsequent to *Clostridium* settlement (Fig.6). Moreover, bacteria belonging to Clostridia-class have notably an acetogenic metabolism. They are capable of simultaneous consumption of hydrogen and carbon dioxide, producing acetate [44]. The internal presence of Clostridia in EABs could then allow partial recovery of a supplied substrate, promoting access to acetate for electroactive species (species tightly attached to the electrode may have restricted access to nutrients once the biofilm is thickened because the substrate will be consumed by the outer microbial layers). It is also known that saccharolytic Clostridia in presence of electron-consuming bacteria, tends to favor metabolic pathways that produce acetate [45] and thus participate in enhanced conservation of EABs performance. But, acetate production alone could not explain *Geobacter spp* dominance because both *Geobacter* spp. and *Desulfuromonas* spp. can use acetate as electron donor. However, bacteria belonging to *Clostridium* genus are known to be fermentative forming hydrogen bacteria [46], and some species belonging to *Geobacte*r are able to recycle hydrogen as electro donor while *Desulfuromonas* cannot [37,47]. Our results also suggest a competition between *Geobacter* spp. and *Desulfuromonas* spp. colonizing the biofilm. A recent study showed a specific selection of *Geobacter* spp. when acetate was reduced, depending on the nature of electrode [37,48]. In the present study, the concentration of acetate was controlled at 20 mM all over the time and all experiments were conducted on the same conditions. Thus, the access to acetate could not be the important factor of *Geobacter* pre-dominance in enriched conditions. *Geobacter* spp. is the most dominant and frequently identified electroactive bacteria. In some cases, where *Geobacter* spp. is absent from biofilms it can be replaced by other iron- and sulfate-reducing bacteria [29]. In raw paper mill effluent, *Geobacter* is not found, whereas *Desulfuromonas* is the pre-dominant species [49]. Our results show currents decreased efficiency when *Desulfuromonas* spp. and *Geobacter* spp. co-exist. Results in Microbial fuel cells (MFC) using waste-water treatment show an efficient Desulforomonaceae-dominated biofilm, while non-efficient MFCs showed a co-dominance of the anodic biofilm by both Desulfuromonadaceae and Geobacteraceae [50]. Thus, there seemed to be tough competitive interactions between Desulfuromonadaceae and Geobacteraceae that strongly affect the electroactive efficiency of studied biofilms. This competition was confirmed by a low Pearson's correlation coefficient between *Desulfuromonas* spp. and *Geobacter* spp. (Table 2).

Table 2. Pearson correlation coefficients for the relation of interpresence of bacteria and for the relation of bacterial presence with coulombic efficiency characteristic.

Criteria	D	P	A	N	G	C	D	G
Desulfuromonas (D)								
Pelobacter (P)	0.4							
Actinobacteria (A)	0.5	0.8						
NB1-n (N)	0.4	0.2	-0.1					
Geobacter (G)	-0.1	-0.1	-0.1	0.1				
Clostridium sensu stricto (C)	-0.1	-0.4	0.0	-0.2	0.8			
Desulfovibrio (D)	-0.2	0.1	-0.2	0.4	-0.3	-0.4		

Geopsychrobacter (G)	0.2	0.6	0.2	0.7	-0.1	-0.4	0.6	
Coulombic efficiency (CE)	0.2	-0.3	-0.5	0.6	0.0	-0.1	0.4	0.3

On the other hand, minor bacteria should have a role in modulating electroactivity. In mixed cultures, different co-existing species have specific nutritional requirements and functional roles [51]. A diverse bacterial community could reach a high current density [52,53]. Interestingly, another study showed significant discrepancies in major electroactive species abundances with no influence on bioanode performance [54]. Therefore, it was proposed that the association of different microbial species was a key bioprocess for establishing an efficient community for electron transfer to the anode. The present study confirms the latter hypothesis because no correlation was found between the abundances of electroactive species *per se* and the performance of the three-electrode devices.

In our study, the role of minority bacteria species from mangrove microbial community that served as an inoculum has been highlighted in electroactive biofilms thanks to enrichment. Community analysis disclosed a syntrophic association among *Clostridia* and *Geobacter* species for bioelectricity generation. The statistical analysis showed that minor species of bacteria could have greater influence on the overall efficiency of bioelectrochemical systems than major referenced electroactive species such as *Desulfuromonas* or *Geobacter*. The community patterns aforementioned were read in conjunction with Pearson's correlation coefficient. The reasonable inference of this analysis is that two species of bacteria closely related phylogenetically, present similar metabolic activities, could have a competition for the same nutritional resources and therefore not likely to coexist in a single community. Thus, the minor bacteria in a consortium seem to play a specific role leading to better stability and survival of the community. The diversity of electroactive species in biofilms allowed the formation of different efficient consortia, when changes in their composition induced a complete remodeling of the community. In the current study, it was shown that the relative abundance of the major strictly speaking electroactive bacteria didn't seem to influence bioanode performance. The association of different bacterial taxa would enable a functioning optimization of the electroactive biofilm community. The relationships between the electroactive bacteria themselves and other bacterial species of the community would then be a key factor for the sustainability of efficient biofilms. This study revealed complex relationships and dependences between EABs and their direct environment. Changes in consortia composition induced a remodelling of the overall community involving qualitative and quantitative rearrangements of species, which are not likely to lead the loss of biofilms electroactivity.

5. Conclusion

Our study provides new insight in the potential role of minor bacteria in the overall efficiency of bioelectrochemical systems and in the syntrophic association for bioelectricity generation that should be investigated deeply. Our results constitute a base for further studies dealing with marine mangrove biofilms in bioelectrochemical systems. These microorganisms can cooperate synergistically to increase power production, and this synergism can be exploited as a future alternative in energy production.

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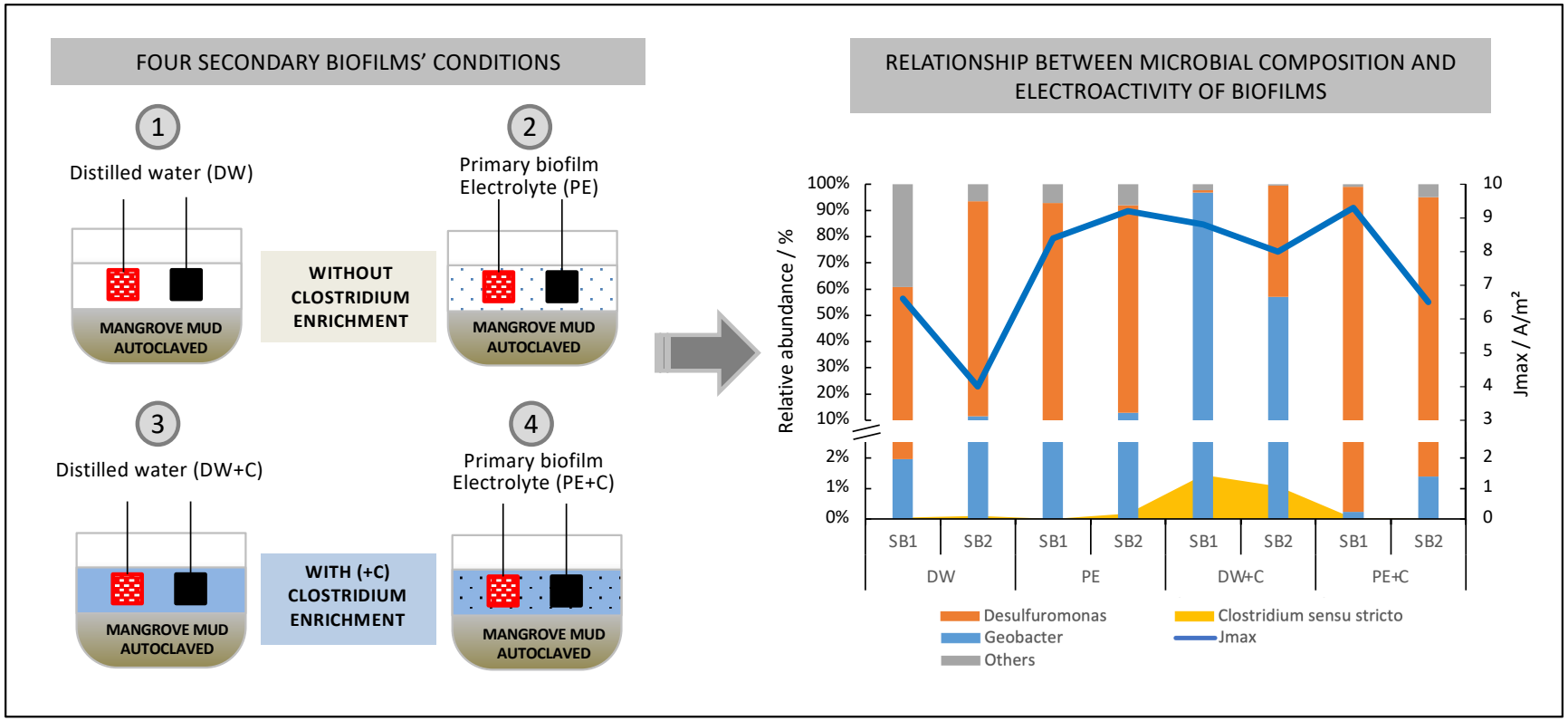
FOUR SECONDARY BIOFILMS' CONDITIONS
1
Distilled water (DW)
MANGROVE MUD AUTOCLAVED
WITHOUT CLOSTRIDIUM ENRICHMENT
2
Primary biofilm Electrolyte (PE)
MANGROVE MUD AUTOCLAVED
3
Distilled water (DW+C)
MANGROVE MUD AUTOCLAVED
WITH (+C) CLOSTRIDIUM ENRICHMENT
4
Primary biofilm Electrolyte (PE+C)
MANGROVE MUD AUTOCLAVED
RELATIONSHIP BETWEEN MICROBIAL COMPOSITION AND ELECTROACTIVITY OF BIOFILMS
Relative abundance / %
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
2%
1%
0%
Jmax / A/m²
10
9
8
7
6
5
4
3
2
1
0
SB1
SB2
DW
PE
DW+C
PE+C
Desulfuromonas
Geobacter
Others
Clostridium sensu stricto
Jmax