COUPLAGE FERMENTATION / ELECTROLYSE MICROBIENNE : INFLUENCE DES ESPECES EXOGENES SUR LE BIOFILM ELECTROACTIF.

Les chapitres précédents ont principalement permis de montrer (1) la faisabilité de réaliser la fermentation en milieu salin à pH 6 et pH 8 et (2) la forte sélection d'espèces électroactives sur anode ou en enrichissement liquide sur Fe(III). Après établissement d'un biofilm électroactif, l'ajout de biomasse fermentaire issue de la fermentation dans le compartiment anodique peut également constituer une perturbation biotique sur le biofilm. Dans ce dernier chapitre nous nous intéressons au couplage de la fermentation en milieu salin à des pH variant de 6 à 8 et de l'électrolyse microbienne (opérée à pH 7). Plus particulièrement, nous étudions ici les interactions entre les deux communautés microbiennes propres à chacun de ces procédés. L'impact d'un ajout de biomasse issue de la fermentation sur le biofilm électroactif a été étudié en termes de transfert d'électrons et de dynamique des populations microbiennes du biofilm électroactif.

V.1. AVANT-PROPOS

Le but de ce travail de thèse a été d'étudier l'impact de conditions de conduite de procédés de fermentation et d'électrolyse microbienne en termes de performance du procédé et de sélection/changement de populations microbiennes. L'originalité de cette étude a été de travailler en milieux salin dans le but d'augmenter la conductivité du milieu en électrolyse microbienne et, de là, améliorer le transport de charges dans l'électrolyte de la cellule d'électrolyse microbienne (Lefebvre et al., 2012).

Les conditions favorables à la production d'hydrogène par fermentation sont communément un pH bas (5-6) et une température de 37°C (Guo et al., 2010). L'électrolyse microbienne est quant à elle réalisée à pH neutre ou alcalin (Liu et al., 2010) et une forte conductivité du milieu est favorable au transport de charges dans l'électrolyte.

Les deux premiers chapitres de résultats de cette thèse portent sur l'étude des variations de ces paramètres et de la faisabilité des procédés de fermentation et d'électrolyse microbienne dans des conditions intermédiaires permettant la conduite de chacun des procédés. Le Chapitre III de cette thèse porte sur l'étude de la fermentation dans des conditions salines et à pH 6 ou 8. Cette partie a notamment permis de montrer la faisabilité de la fermentation en milieu salin à pH 8 avec la sélection de bactéries appartenant à la famille des *Vibrionaceae* et une inhibition de la consommation d'hydrogène par la voie propionate. Le Chapitre IV a quant à lui permis de montrer la forte sélection d'espèces électroactives dans le biofilm en conditions salines en utilisant des sédiments salins comme inoculum. Les biofilms électroactifs obtenus à partir de ces sédiments ont permis d'atteindre de fortes densités de courant et des rendements faradiques élevés. Néanmoins, la faible diversité microbienne du biofilm électroactif conduit une certaine fragilité de la stabilité de l'écosystème. En effet, la robustesse des biofilms et leur résistance aux perturbations externes réside principalement dans leur diversité (Jouenne, 2008). Le couplage de la fermentation avec l'électrolyse microbienne peut induire des perturbations abiotiques telles que la composition du milieu, le pH, la température etc… mais aussi biotiques avec l'ajout d'espèces exogènes issues du fermenteur (*Figure V-1*).

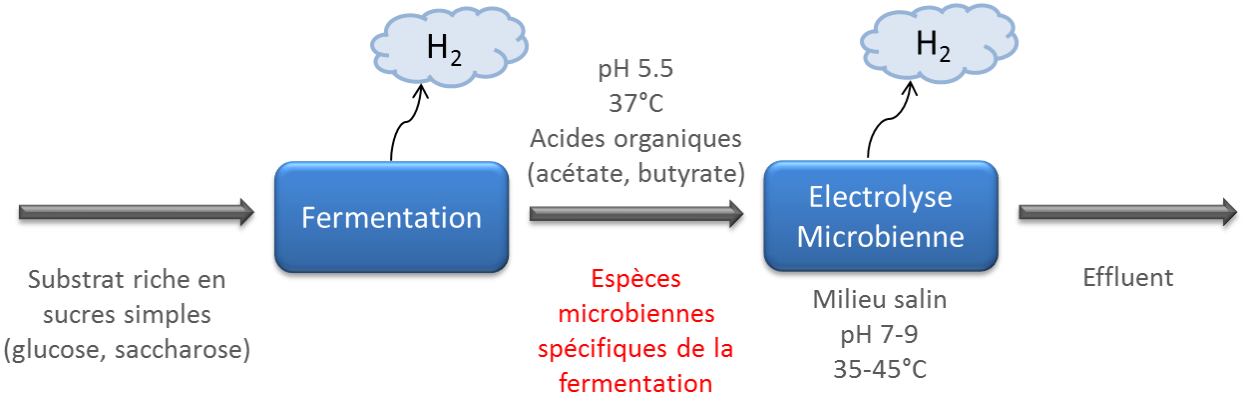


Figure V-1 : Paramètres spécifiques liés au procédé de fermentation et d'électrolyse microbienne dans le cadre du couplage.

Le biofilm électroactif peut être affecté dans son fonctionnement et sa structure de communauté microbienne par des perturbations abiotiques mais aussi à des perturbations biotiques liées à l'apport d'espèces spécifiques dans le compartiment anodique de la cellule d'électrolyse microbienne

Néanmoins, les précédentes études portant sur le couplage des deux procédés ont montré sa faisabilité (Lalaurette et al., 2009; Lu et al., 2009; Wang et al., 2011; Tommasi et al., 2012; Lenin Babu et al., 2013). Ces études rapportent des rendements globaux de conversions du système combiné fermentation-électrolyse microbienne jusqu'à 9,95 $mol_{H2}/mol_{glucose}$ (Lalaurette et al., 2009) et 8,79 $mol_{H2}/mol_{glucose}$(Lu et al., 2009). Cependant, la majeur partie de ces études ont été conduites en s'affranchissant du facteur biotique de l'apport en micro-organismes fermentaires par centrifugation des effluents de fermentation pour séparer le milieu liquide des microorganismes avant alimentation de l'électrolyseur (Lalaurette et al., 2009; Lu et al., 2009; Wang et al., 2011). Bien que les changements de conditions expérimentales en passant du fermenteur à l'électrolyseur puissent affecter ces bactéries exogènes, leur introduction dans le compartiment anodique peut également impacter la structure des communautés microbiennes au sein du biofilm ainsi que ses performances d'électroactivité. A notre connaissance, cet aspect n'a été étudié que dans une seule étude (Lenin Babu et al., 2013) où la présence de γ-protéobactéries (50%), bacilles (25%) et clostridies (25%) a été noté dans le compartiment anodique après couplage. L'identification de clostridies montre la présence et la survie de bactéries potentiellement issues de la fermentation. Cependant, l'influence de bactéries exogènes issues de la fermentation sur le biofilm en électrolyse microbienne n'est pas décrite dans la littérature

en termes d'impact sur l'activité du biofilm et sur la dynamique des communautés microbiennes.

L'objectif de l'étude présentée dans ce chapitre a été de déterminer si la biomasse issue de l'étape de fermentation pouvait avoir un impact sur le biofilm électroactif. L'article s'attache particulièrement à décrire (1) la dynamique de structure des communautés microbiennes du biofilm après ajout d'espèces exogènes au cours de batchs successifs et (2) les modifications de l'activité électroactive du biofilm. Trois biomasses issues d'effluents de fermentation réalisées dans des conditions de pH différentes ont été utilisées. La biomasse fermentaire a été obtenue à partir de fermenteurs en batch réalisés en milieu salin (suite aux résultats du Chapitre III) à pH 6, 7 ou 8 correspondant, respectivement, au pH de la fermentation classiquement décrit dans la littérature pour la production d'hydrogène (Guo et al., 2010), au pH de l'électrolyse microbienne (Logan, 2012) et au pH de l'inoculum ainsi qu'à celui de la fermentation réalisée dans le Chapitre III (Pierra et al., 2013).

Cette étude vise à apporter des réponses à la question suivante : les performances électroactives et la structure de communauté microbienne sont-elles affectées par l'ajout d'espèces exogènes issues de la fermentation?

V.2. COUPLAGE FERMENTATION / ELECTROLYSE MICROBIENNE : INFLUENCE DES ESPECES EXOGENES SUR LE BIOFILM ELECTROACTIF

Influence of dark fermentative biomass on electroactive biofilm functionality and microbial community composition.

PIERRA Mélanie, RIVALLAND Caroline, TRABLY Eric, GODON Jean-Jacques, BERNET Nicolas

Cet article est en cours de préparation. Des résultats complémentaires sont attendus et il ne sera pas soumis en l'état.

Abstract :

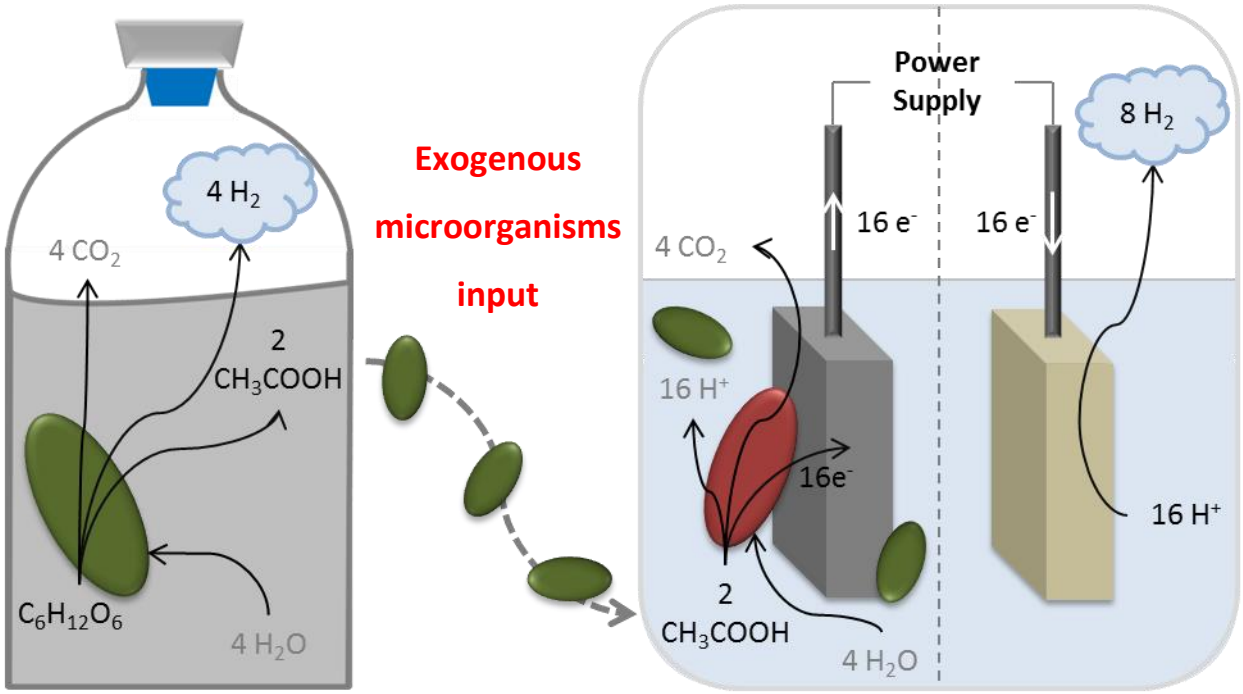
Biohydrogen production is of increasing interest in a context of fossil fuel increasing deficiency. Dark fermentation process is limited to a theoretical conversion yield of 4 $mol_{H2}/mol_{Glucose}$ with concomitant production of organic acids such as acetate and butyrate. Microbial electrolysis completely oxidizes organic matter into carbon dioxyde and hydrogen using electrochemical biofilms with a theoretical yield of 8 $mol_{H2}/mol_{Glucose}$. The association of the two processes would permit to convert all the disposable hydrogen of organic matter. So far, the dynamic of the impact of exogenous bacteria from fermentation process on the electroactive biofilm bacterial community of microbial electrolysis has not been further studied. This research aims at establishing whether electron transfer and bacterial community structure of the electroactive biofilm could be affected by bacteria originated from dark fermentation processes.

For this, a multi-electrode reactor was used. Electrodes were removed successively to characterize the electroactive performances and the bacterial community of biofilm changes among time after exogenous bacteria injection.

The findings of this study suggest that the electroactive biofilm originated from sediment presents a biofilm presenting high current densities up to 10,8±1,3 A/m^2 and a robust bacterial community structure. Exogenous bacteria coming from the batch dark fermenters did not have any effect on the biofilm bacterial structure. Nevertheless, it affected the current density generated by the biofilm likely due to EPS production and methane production occurring in batch conditions that respectively slowed down substrate transfer in the biofilm competed for substrate consumption.

Keywords : Biohydrogen, Microbial electrolysis cell (MEC), Acidogenic effluent, CE-SSCP fingerprinting

Graphical abstract :



V.2.1. INTRODUCTION:

Hydrogen (H_2) as energy carrier is of increasing interest. It is a promising clean and sustainable energy carrier. This is due to its high energy density and its high efficiency as fuel cells for conversion of H_2 to electricity for transportation purpose (Hawkes et al., 2007; Hallenbeck and Ghosh, 2009; Guo et al., 2010). Nowadays, hydrogen is mostly produced by natural gas reforming which causes twice carbon dioxide equivalent compared to biohydrogen and contributes to carbon depletion (Das and Veziroglu, 2001; Hajjaji et al., 2013). However, recent developments in the field of biotechnologies and sustainable development led to a renewed interest in alternative technologies to produce hydrogen from different biomass sources such as dark fermentation and, more recently, microbial electrolysis (Hallenbeck and Benemann, 2002; Logan et al., 2008; Gómez et al., 2011).

The past decades have seen increasing and rapid advances in the field of microbial electrolysis cells (MEC) development. A MEC is a process that permits to convert organic matter into hydrogen. It consists in an anode on which organic substrates are microbiologically oxidized with a release of electrons associated with a cathode that uses the

generated electrons to produce hydrogen following the electrochemical reduction of water (Logan et al., 2008; Liu et al., 2010). In MECs, the oxidation process is achievable because of the development on the anode of a bacterial biofilm which catalyses organic matter oxidation. This electroactive biofilm contains electroactive bacteria, also named anode respiring bacteria (ARB), that have the ability to transfer electrons released from organic matter oxidation to an insoluble and external electron acceptor, e.g. the anode. In comparison to hydrogen production with strictly electrochemical process of water electrolysis, MEC requires 5 to 10 less energy input, decreasing the energetic cost of so produced hydrogen (Liu et al., 2010).

Although microbial characterisation of several microbial BESs already showed the predominance of *Geobacter sulfurreducens* in electroactive biofilms, (Holmes, Bond, O'Neil, et al., 2004; Badalamenti et al., 2013), analysis of the microbial diversity of enriched anodic biofilms revealed that different microorganisms might be responsible for the observed electroactivity. However, these analyses did not reveal any tendency in dominant members within the biofilm communities. An example of such microbial diversity is shown by the ARBs usually found associated to either α, β, γ, δ-*Proteobacteria*, *Firmicutes*, *Acidobacteria* or *Actinobacteria* (Liu et al., 2010).

Biohydrogen production process by dark fermentation pathways in mixed cultures allows the conversion of hexose (saccharose, glucose) with limited conversion rate (2-3 moles of hydrogen per mole of hexose) and with the concomitant production of organic metabolites (mainly acetic and butyric acids) (Hawkes et al., 2007; Guo et al., 2010). Fed with acetate, a MEC produces up to 3 moles of hydrogen per mole of acetate (Liu et al., 2010; Gómez et al., 2011). The association of dark fermentation and MEC process could theoretically lead to the production from 8 to 9 moles of hydrogen per mole of hexose, which is close to the maximum theoretical potential hydrogen yield of 12 moles of hydrogen per mole of hexose.

However, environmental conditions are rather different between dark fermentation (low pH in a range of 5-6, 37°C) and MEC (neutral or alkaline pH, high conductivity). Nevertheless, previous studies showed the feasibility of dark fermentation at pH 8 under saline conditions ($35g_{NaCl}/L$) in mixed culture (Pierra et al., 2013). While many species belonging to *Clostridium, Enterobacter* and *Escherichia* genera have been described in the literature as hydrogen-producing bacteria in mixed cultures (Hawkes et al., 2007; Wang and Wan, 2009a, 2009b; Guo et al., 2010; Quéméneur et al., 2010, 2012; Quéméneur, Hamelin, Benomar, et

al., 2011; Quéméneur, Hamelin, Latrille, et al., 2011), Pierra et al. (2013) reported a shift from *Clostridiales* to *Vibrionales* as main dominant bacteria in saline environment at pH 8 with an efficient hydrogen production of 0,90 ±0.02 $mol_{H2}/mol_{Glucose}$ at 75 g_{NaCl}/L.

Other studies considered the association of these two processes (Lalaurette et al., 2009; Lu et al., 2009; Wang et al., 2011; Tommasi et al., 2012; Lenin Babu et al., 2013). Tommasi et al. (2012) compared the two following coupling systems: $BioH_2$+CH_4 (dark fermentation and methane production) and $BioH_2$+MEC (dark fermentation and H_2-producing MEC). The $BioH_2$+MEC system provided better results in terms of energetic conversion with 2.41 $mol_{H2}/mol_{acetate}$ with the MEC, which would correspond to a theoretical additional conversion rate of 4.82 $mol_{H2}/mol_{glucose}$ to dark fermentation. In this study, coupling was just simulated with a MEC fed with acetate and was not actually implemented. Other studies report a significant conversion of organic matter into hydrogen close to theoretical estimations, up to 9.95 $mol_{H2}/mol_{glucose}$ (Lalaurette et al., 2009) and 8.79 $mol_{H2}/mol_{glucose}$(Lu et al., 2009).

So far, there has been little discussion about microbial aspects of such a coupling. Indeed, in most of the studies dealing with the association of dark fermentation and MEC for hydrogen production, effluent from dark fermentation process are centrifuged and the supernatant used to feed the anodic compartment of the MEC in order to avoid any disturbance of the MEC by fermentative bacteria (Lalaurette et al., 2009; Lu et al., 2009; Wang et al., 2011). This additional process implies increase in energy cost of the coupling. Whereas the experimental conditions change between dark fermentation and MEC (neutral pH, high conductivity, nutrient deprivation phenomenon), which could affect the fermentative bacteria, their introduction in the anodic compartment can also impact the ARB community structure and electrogenic activity. To date, this specific microbial interaction aspect has only recently been investigated (Lenin Babu et al., 2013). Lenin Babu et al. (2013) found the presence of *γ-Proteobacteria* (50%), *Bacilles* (25%) and *Clostridia* (25%) in the anodic compartment after coupling a dark fermenter and a MEC. Identification of *Clostridia* sp. shows the presence and the survival of fermentative bacteria in the anodic compartment. However, data are missing to better describe the influence of exogenous bacteria from dark fermentation process on microbial communities in terms of population structure and electroactive performances.

The objectives of this work are to determine whether the microbial biomass from the dark fermentation effluent could impact the activity of the ARB biofilm. This paper will focus on examining the dynamics of microbial community structures in successive batch experiments after introduction of the biomass obtained from dark fermentation performed at pH 6, pH 7 and pH 8. These pH conditions correspond respectively to the pH of dark fermentation (Guo et al., 2010), MEC (Logan, 2012) and to the inoculum or the possible dark fermentation process pH (Pierra et al., 2013).

The main issues addressed in this paper are: Are the electron transfer performances and the bacterial community structure of the ARB biofilm affected by exogenous bacteria issued from the dark fermentation process operated under three different pH conditions?

V.2.2. MATERIALS AND METHODS:

Fermentative biomass was grown and collected from dark fermentation batch experiments at three different pH values: 6, 7 and 8. Then, the potential impact of these biomasses on an anodic biofilm in terms of bacterial community structure and performances was assessed using four different devices as described below. Four bioelectrochemical reactors were operated at pH 7, usual pH in BES (Logan, 2012). One was conducted at pH 7 with no addition of exogenous biomass as a control (RBC), and the three others were carried out with addition of the biomass from the dark fermenter operated at pH 6 (RB6), pH 7 (RB7) or pH 8 (RB8).

V.2.2.1. SOURCE OF INOCULUM

The seed sediment used for hydrogen production in dark fermenter and biofilm growth in MEC was the same. It was sampled in a lagoon collecting wastewaters from a salt factory (Gruissan, France). The sediments were stored at lab temperature before inoculation. The initial pH of the sediments was 8.5 and the salinity 67.4 g_{NaCl}/L.

V.2.2.2. BIOMASS COLLECTION FROM HYDROGEN-PRODUCING BATCH TESTS

For each pH condition of biomass formation in batch cultures, hydrogen production experiments were performed in ten different 600 mL glass bottles. About 2.6 g of the seed

sediment was added to the culture medium to obtain a final concentration of 450 mgVS /L (final working volume of 200 mL). The culture medium was composed of 50mM MES (2-(N-morpholino)ethanesulfonic acid) buffer, 5 g/L glucose and a mineral solution (Rafrafi *et al.*, 2013) (0.5g/L K_2HPO_4, 2g/L NH_4Cl, 0.2g/L Yeast Extract, 55mg/L $MgCl_2$, 6H2O, 7mg/L $FeSO_4(NH_4)2SO_46H_2O$, 1mg/L $ZnCl_2$, 2H2O, 1.2mg/L $MnCl_2$, $4H_2O$, 0.4mg/L $CuSO_4$,5 H_2O, 1.3mg/L $CoSO_4$, $7H_2O$, 0.1mg/L BO_3H_3, 1mg/L $Mo_7O_{24}(NH_4)_6$, $4H_2O$, 0.05mg/L $NiCl_2$, 6 H_2O, 0.01mg/L Na_2SeO_3, 5 H_2O, 60mg/L $CaCl_2$, 2 H_2O, 35 g/L NaCl). The initial pH was adjusted to 6, 7 or 8 using NaOH (1M). To ensure anaerobic conditions, each bottle was flushed after inoculation with nitrogen gas for 15 minutes. Then, the bottles were capped with a rubber stopper and incubated at 35°C until they reached the maximum accumulated volume of hydrogen.

Biogas production was periodically estimated by measuring the headspace pressure. Biogas composition (CH_4, CO_2, H_2 and N_2) was determined using a gas chromatograph (Clarus 580, Perkin Elmer) coupled to Thermal Catharometric Detection (TCD), as described elsewhere (Quéméneur et al., 2012).

The biomass was collected and centrifuged at 20,000 g for 30 minutes. Biomass was collected and resuspended in the original mineral medium (with no glucose). 40 mL of this biomass were added to the N-Stat reactor (OD_{620nm}= 16.6±0.8)

Aliquots of two-milliliters were collected at the end of each cycle and centrifuged (20,000g, 10 min). Supernatants and pellets were stored at -20°C. Supernatants were used for further chemical analysis and pellets for DNA extraction.

V.2.2.3. Bio-Electrochemical Systems (BES)

V.2.2.3.1. *Medium for BES operation*

To be prepared to the coupling, the culture medium was the same as described previously in H_2 production batch tests operated for biomass collection, except that glucose as carbon source was replaced by 10 mM of acetate per graphite electrode present in the reactor.

Inoculum was added into culture medium (10% v/v for a final working volume of 500mL). To ensure anaerobic conditions, all reactors were flushed with nitrogen gas for 30 minutes after inoculation. Reactors were incubated at 37°C.

V.2.2.3.2. *Bioelectrochemical arrangement set up*

The devices used for the development of bacterial biofilms consisted of an arrangement of 6 electrodes including a reference electrode, a counter electrode and 4 working electrodes. All working electrodes were connected to the same reference and auxiliary electrodes using a multi-channel potentiostat equipped with a NStat system (VMP Bio-Logic SA). The electrodes corresponded to 4 graphite working electrodes (WE) 2.5x2.5x0.2cm (Goodfellow), a platinum Iridium grid (90%/10%) (Heraeus) as a counter electrode (CE) 5x5cm (Goodfellow) and a reference electrode (Saturated Calomel Electrode - SCE). To ensure the electrical connection with the working electrodes, titanium rods of 2 mm diameter and 12 cm long (Goodfellow) were used. The working electrodes were cleaned before use using a P800 sandpaper and rinsed with 99% purity ethanol. The platinum iridium grid cathode/or counter electrode was cleaned by heating in a blue flame. This system was particularly interesting as it permitted the simultaneous development of four identical electroactive biofilms using the four working electrodes as final electron acceptors.

To ensure anaerobic conditions, all reactors were flushed with nitrogen gas for 30 minutes after inoculation. Reactors were incubated at 37°C. The anode potential was fixed at +0.2V vs SCE (potential +0.244 vs. SHE). Reactor medium was continuously homogenized thanks to a magnetic stirrer used at a 300 round per minute speed.

Figure V-2 presents the N-Stat disposal used to get replicate biofilms on graphite plates. The 4 electrodes were successively removed from the disposal to follow the evolution of biofilm microbial community structure.

Current densities (j in A/m²) of the microbial biofilms were calculated considering the total immersed electrode surface area since electroactive biofilms covered both sides of the electrodes with the same microbial community profile (CE-SSCP patterns). Coulombic efficiencies (C_E in %) were calculated for each experiment according to Call *et al.* (2009).

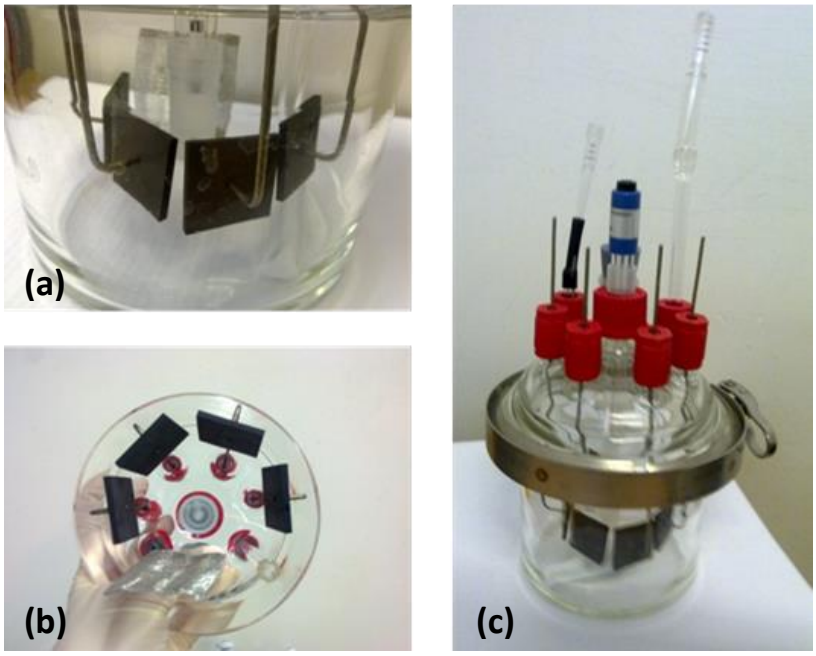


Figure V-2 : N-Stat arrangement to grow electroactive biofilms.

This disposal contains 4 graphite working electrodes, a platinum grid as a counter electrode and a reference electrode (Saturated Calomel Electrode - SCE). (a) View of the 4 graphite electrodes facing the SCE reference electrode and the platinum grid, (b) bottom view of the electrodes arrangement, (c) overall view of the empty reactor containing the 6 electrodes, a liquid and a gas sampling devices.

V.2.2.4. CHEMICAL ANALYSES

VFA composition of acetic (C2), propionic (C3), butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6) acids, was determined using a gas chromatograph (GC-3900 Varian) equipped with a flame ionization detector. Concentrations of non-VFA metabolic products such as ethanol, lactate and formate were measured by HPLC analysis and refractometric detection as described elsewhere (Quéméneur et al., 2012). Biogas production was periodically estimated by measuring the gas pressure in headspace. Biogas composition (CH_4, CO_2, H_2 and N_2) was determined using a gas chromatograph (Clarus 580, Perkin Elmer) coupled to Thermal Catharometric detection (TCD), as described previously (Quéméneur et al., 2012).

V.2.2.6. MOLECULAR ANALYSES

V.2.2.6.1. *DNA extraction, PCR amplification and CE-SSCP fingerprinting*

DNA from biofilms was first extracted. Microbial community of biofilms and inoculum were characterized using capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) and sequenced using pyrosequencing. Molecular analyses of bacterial communities were performed on each electroactive biofilm. Genomic DNA was extracted and purified using a previously described protocol (Godon *et al.* 1997). Total extracted DNA was purified using a QiAmp DNA microkit (Qiagen, Hilden, Germany). Extracts amount and purity of DNA were confirmed by spectrophotometry (Infinite NanoQuant M200, Tecan, Austria). Then, the 16S rRNA genes were amplified using universal primers for bacteria, named W49 (5'-ACGGTCCAGACTCCTACGGG-3' *Escherichia coli* position 330) and 5'-fluorescein phosphoramidite labeled W104 (5'-6FAM-TTACCGCGGCTGCTGGCAC-3' *Escherichia coli* position 533), according to Wéry (2008). Each PCR mixture (50µL) contained 5µL of 10x Pfu Turbo DNA buffer, 200 nMf of dNTP, 500 nMf of each primer, 2.5 U μl^{-1} of Pfu Turbo DNA polymerase (Stratagene) and 10 ng of genomic DNA. Reactions were performed in a Mastercycler thermal cycler (Eppendorf). The 16S rRNA genes were amplified as follows: initial denaturing step at 94°C for 2 min, followed by 25 cycles performed at 94°C for 30 s, 61°C for 30 s and 72°C for 30 s, with a final elongation at 72°C for 10 min. Reactions were stopped by cooling the mixture to 4°C.

A capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) method was used for PCR products fingerprinting. CE-SSCP is a method that separates DNA fragments with the same size but having different secondary conformation rearrangement according to their base compositions (Wéry *et al.* 2008). To perform CE-SSCP fingerprinting, one microliter of the appropriate dilution of PCR products was mixed with 18.925 µL of formamide and 0.075 µL of internal standard GeneScan ROX (Applied Biosystems). Samples were heat-denatured at 95°C for 5 min and re-cooled directly in ice for 5 min. CE-SSCP electrophoresis was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems) in 50 cm capillary tubes filled with 10% glycerol, conformation analysis polymer and corresponding buffer (Applied Biosystems). Samples were eluted at 12kV and 32°C for 30 min, as described elsewhere (Wéry *et al.* 2008).

CE-SSCP profiles were aligned with an internal standard, ROX, to consider the inter-sample electrophoretic variability. The CE-SSCP profiles were normalized using the StatFingerprints library (Michelland *et al.* 2009) in R software version 2.9.2 (R. Develpment Core Team, 2010), with a standard procedure described elsewhere. (Fromin *et al.* 2007). Simpson diversity index (Simpson, 1949) was evaluated to estimate the complexity of the community by assessing the number of species (number of peaks) as well as the relative abundance (area under each peak) (Quéméneur *et al.* 2011).

One DNA samples, pyrosequencing was performed using a 454 protocol for bacterial identification (Research and Testing Laboratory (Lubbock, USA)).

V.2.2.6.2. *qPCR*

PCRs were prepared using 96-well real-time PCR plates (Eppendorf, Hamburg, Germany) and Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). Then, 12.5 µl of Express qPCR Supermix with premixed ROX (Invitrogen, France), 5 µl of DNA extract with three appropriate dilutions, 10 nM forward and reverse primers, 8 nM TaqMan probe, and water were added to obtain a final volume of 25 µl for all analyses An initial incubation of 20 s at 95°C and 40 cycles of denaturation (95°C, 15 s; 60°C, 1 min) were performed. One standard curve was generated from each assay by using 10-fold dilutions in sterilized water (Aguettant Laboratory, Lyon, France) of the PCR products from known environmental clones. Clone LC103 was used as standard for Bacteria,. The initial DNA concentrations were quantified using the Infinite 200 PRO NanoQuant (Tecan, France). Two measurements were obtained per sample for each primer set.

V.2.3. RESULTS AND DISCUSSION:

V.2.3.1. EXOGENOUS BIOMASS FROM DARK FERMENTATION

V.2.3.1.1. *Hydrogen production in batch tests*

Throughout the batch experiments, no CH_4 was detected in the headspace and only H_2 and CO_2 were found as gaseous products. The maximum hydrogen production yields were 0.469±0.209 $mol_{H2}/mol_{Glucose}$, 0.510±0.096 $mol_{H2}/mol_{Glucose}$ and 0.517±0.158 $mol_{H2}/mol_{Glucose}$ at pH of 6, 7 and 8, respectively (*Figure V-3*). These results are in good agreement with previous results published on dark fermentation (Quéméneur et al., 2012; Pierra et al., 2013). As an illustration, Pierra et al. (2013) obtained 0.65±0.04 $mol_{H2}/mol_{Glucose}$ in dark fermentation batch reactors conducted under saline conditions at pH 8 and using a saline sediment inoculum.

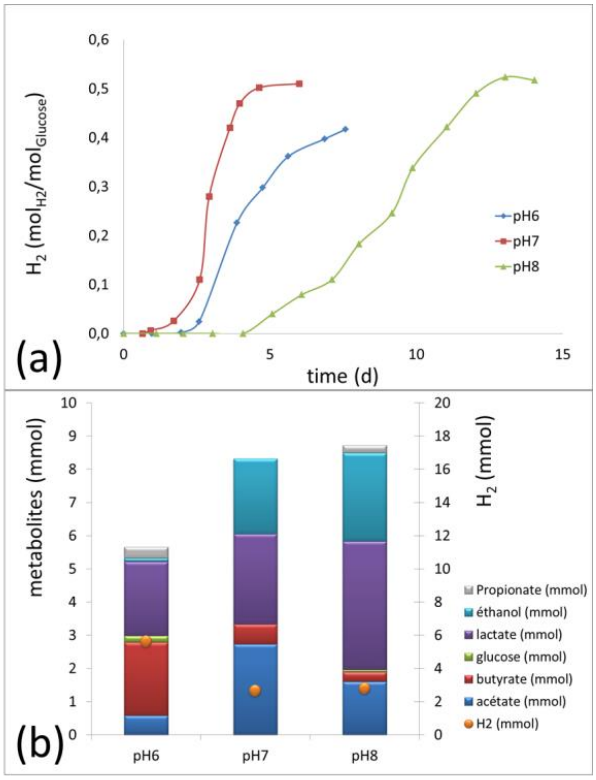


Figure V-3 : Hydrogen production of biomass used as exogenous bacteria on biofilm at the maximum hydrogen production point.

(a) Hydrogen production yield (molH2/molGlucose) for each series of batch of fermentation reactor at pH 6, pH 7 and pH 8. (b) Metabolic end-products (in mmoles) and maximum H2 production (in mmoles) according to pH. The values correspond to an average of batch replicates.

In the batch tests operated at pH 8, a substantial increase of the lag phase occurred from 1.4±0.7 days (pH 6-7) to 4 days. A possible explanation for this increase could be either a low amount of microorganisms capable of hydrogen production in the inoculum, a longer generation time of these microorganisms or an osmotic adaptation of the microorganisms (Oren, 2001; Van Niel et al., 2003).

The metabolic end products generated by fermentation at the maximum hydrogen production point are reported on *Figure V-3*(b). The production of hydrogen occurred via acetate and butyrate pathways. However, concomitant concurrent routes inhibited the hydrogen conversion rate (ethanol and lactate). Interestingly, the low quantity of propionate measured in all batch reactor corroborates the hypotheses of Pierra et al. (2013) who suggested that hydrogen consuming propionate-producing bacteria were strongly inhibited by NaCl.

V.2.3.1.2. *Microbial community*

The 16S rRNA gene based CE-SSCP profiles of cultures operated at different pH are presented in Figure V-4. Each profile corresponds to a mix of batch bottles' bacterial communities at the maximum hydrogen production time. In comparison with the inoculum Simpson diversity index of 0.99, these profiles presented very simple communities structures with diversities indexes of 0.95, 0.91 and 0.86 (respectively for pH 6, pH 7 and pH 8). Moreover, a strong bacterial selection appears with only one or two main peaks as dominant bacteria for each pH batch communities. These results further support the idea of a high selection in bacterial communities due to dark fermentation process (Quéméneur, et al., 2011; Pierra et al., 2012; Quéméneur et al., 2012)

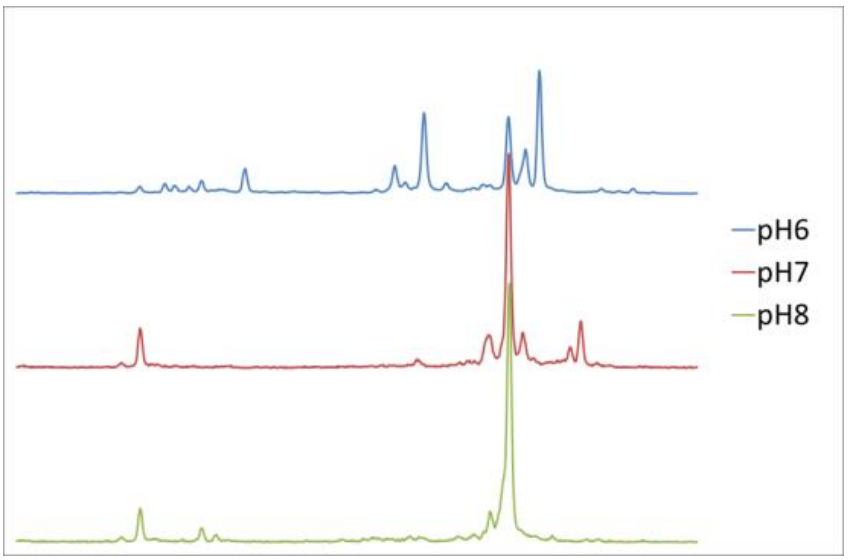


Figure V-4 : CE-SSCP profiles of biomass based on 16S rRNA gene fragments retrieved from fermentation batches at pH 6, pH 7 and pH 8.

The profiles correspond to samples taken at the maximum hydrogen production time. X and Y axes represent respectively the relative peak electrophoresis migration distance and the relative peak intensity (Arbitrary units).

V.2.3.2. EFFECT OF EXOGENOUS BACTERIA ON ANODIC BIOFILM

V.2.3.2.1. *Effect on electron transfer*

All reactors were operated at pH 7. No biomass addition was operated in reactor RBC. The three others reactors were conducted with the addition of biomass from dark fermentation batch tests operated at pH 6 (RB6), 7 (RB7) or 8 (RB8). Figure V-5 presents the current densities among time (chronoamperometric curves) for all reactors with removal time for each biofilm and exogenous biomass addition time. On all graph (Figure V-5), each cycle starts with acetate addition and correspond to all acetate consumption. Current density is assessed to figure out the electron transfer rate to the anode. A conductive biofilm would permit to reach high current densities (Lovley, 2012). The chronoamperometric curves on first cycles of each reactor present a good reproducibility of biofilm performances with the N-stat device. Tableau *V-1* presents the Coulombic efficiency (C_E) as well as the maxima current densities values (J_{max}). C_E depicts the fraction of electrons available in the substrate that ends up as electrical current in the system (Sleutels et al., 2011). This parameter indicates the percentage of electrons harvested from the oxidation of substrate and transferred to the anode by the biofilm. An increase of the Coulombic efficiency is consequently one of the most important challenges in BESs, since as much as possible of the available energy (electrons) from the organic substrate has to be transferred to the anode (Hamelers et al., 2010) .

With no biomass addition (RBC), a conservation of the current densities was shown along the cycles with stable maximum currents densities from 9.3 ±0.1 A/m² for the first cycle to 9.2±0.6 to the third cycle (Figure V-5, Tableau *V-1*).

Addition of biomass from batch fermenters performed at pH 6 in RB6 resulted in a decrease of the maximum current density from 9.8±0.8 A/m² to 5.7±0.1 A/m² for the first and third cycles, respectively (Figure V-5, Tableau *V-1*). Reactor RB8, in which the biomass from the batch fermenters performed at pH 8 was added, presented the same behavior with a decrease of the maximum current density from 9.6±0.8 A/m² to 5.4±0.5 A/m² from the first to the last cycle (Figure V-5, Tableau *V-1*). However the Coulombic efficiency was maintained in both reactors. This is consistent with a previous study of Lu et al. (2009) that operated a single chamber MEC with centrifuged and non-centrifuged effluent from ethanol dark fermentation to determine whether the microbial biomass and other particulate COD

affected hydrogen production. At an applied voltage of +0.6 V vs. Ag/AgCl, they obtained hydrogen production rates of 1.39m^3 H_2/m^3.d (135A/m^3) using a centrifuged sample, compared with 1.32 m^3 H_2/m^3.d (123A.m^3) with a non-centrifuged sample. This suggested that substrate conversion into electrons was not affected by the exogenous biomass. In the specific case of our study, the batch conditions could be the cause of the decrease of the current densities. Indeed, previous studies suggested that batch conditions advantage methanogens that requires a longer lag phase (Lee et al., 2009). Indeed, we measured methane in all ends of batch 3rd cycle with 33.4±9.7% CH_4.

In RB6 and RB8, the decrease of the electron transfer rate of the biofilm (j_{max}) was likely due to an increase of the biofilm thickness, as a thicker biofilm was observed. These simultaneous phenomena seemed to be linked and be related to the fact that the biofilm thickness was responsible for the decrease of the electron transfer rate. What can be hypothesized is that the stress provoked by the addition of exogenous bacteria species on the indigenous species developed on the anode could have activated metabolism pathways for the production of EPS (extracellular polymeric substances). The possible synergistic interactions between bacteria composing the mixed culture biofilm could explain the resistance observed in opposition to the different exogenous species added into the bioreactor where the electroactive biofilms could develop. Indeed, Burmølle et al. (2006) demonstrated that multispecies biofilms characterized with bacterial synergistic interactions were producing a greater amount of biomass and showing a particular resistance to bacterial invasion. These observations fit with the increase in biofilms thickness observed after the exogenous contamination but also regarding the resistance to any exogenous settlement in the biofilm structure. Thus, an increase of the biofilm thickness could be the cause of a more difficult access to the substrate and/or a complicated and longer transfer of the electrons from the outside bacteria layers of the biofilm to the bacteria layer in contact with the electrode, finally transferring the electrons. A thicker biofilm can be the place for the substrate sequestration. Indeed Freguia et al. (2007) showed that electroactivity of MFCs biofilms can be maintained after the primary substrate has been reduced to low concentrations in liquid phase, suggesting a crucial role of substrate storage in the thicker EPS matrix of the biofilm. This way, the acetate located in the extracellular matrix can only be used by electroactive bacteria and not by the suspended/planktonic ones.

The addition of the microbial biomass issued from the batch tests performed at pH 7 in RB7 showed an increase of the maximum current density in the second cycle (just after exogenous bacteria addition). This increase was likely due to a less important perturbation of biofilm with an exogenous biomass originating from a batch fermenter run at the same pH as the BES system. It could also be due to a technical constraint that caused the removal of 2 electrodes and provided more substrate (quantity added for 3 electrodes) to the 2 remaining electrodes. This higher substrate concentration in the reactor, compared to RB6 and RB8, could cause a higher current density (Torres et al., 2007). Then, the maximum current density of RB7 reactor decreased sharply from 10,8±1,3 to 4.1 A/m², presenting the same current production curve as RB6 and RB8 (Figure V-5, Tableau *V-1*). As observed for RB6 and RB8, RB7 presented maintenance of Coulombic efficiency (Tableau *V-1*). This supports the previous hypothesis made with RB6 and RB8 of a thicker biofilm limiting the electron transport through biofilm as naked eye observations were similar (thicker biofilm in RB6, RB7 and RB8 than in RBC).

On the other hand, the Coulombic efficiency (C_E) seems to be maintained with the addition of exogenous species. In all reactors, the Coulombic efficiency, higher that 100%, was likely due to the oxidation of hydrogen produced at the cathode that can be operated by electroactive bacteria and produces more electrons sent to the cathode (Lalaurette et al., 2009; Miceli et al., 2012) since a significant percentage of hydrogen was detected in the gas phase of our experiments (e.g., the percentage of hydrogen during chronoamperometry at the maximum of current production was 73.0% in RBC; 67.2% in RB6; 65.1% in RB7 and 53.1 % in RB8).

A significant percentage of methane was observed at the end of the 3rd cycle as well as no hydrogen in the gas phase of our experiments (e.g., the percentage of methane during chronoamperometry at the end of the 3rd cycle was 40.0 % in RBC; 35.1 % in RB6; 19.2 % in RB7; and CH_4 39.2 % in RB8). Hydrogen consumption and CH_4 production were already observed during long batch cycles experiments (Lee et al., 2009). Indeed, methanogens could be brought with the inoculum in bioreactors. These methanogenic *Archaea* could also be present in the biomass coming from the dark fermentation tests. Since the doubling time is longer for methanogenic *Archaea* than for bacteria (Lee et al., 2009), batch conditions were more favourable to methanogens to grow not being washed. Using this property, in

continuous flow reactor, methane production could be avoided with a low hydraulic retention time (Lee et al., 2009).

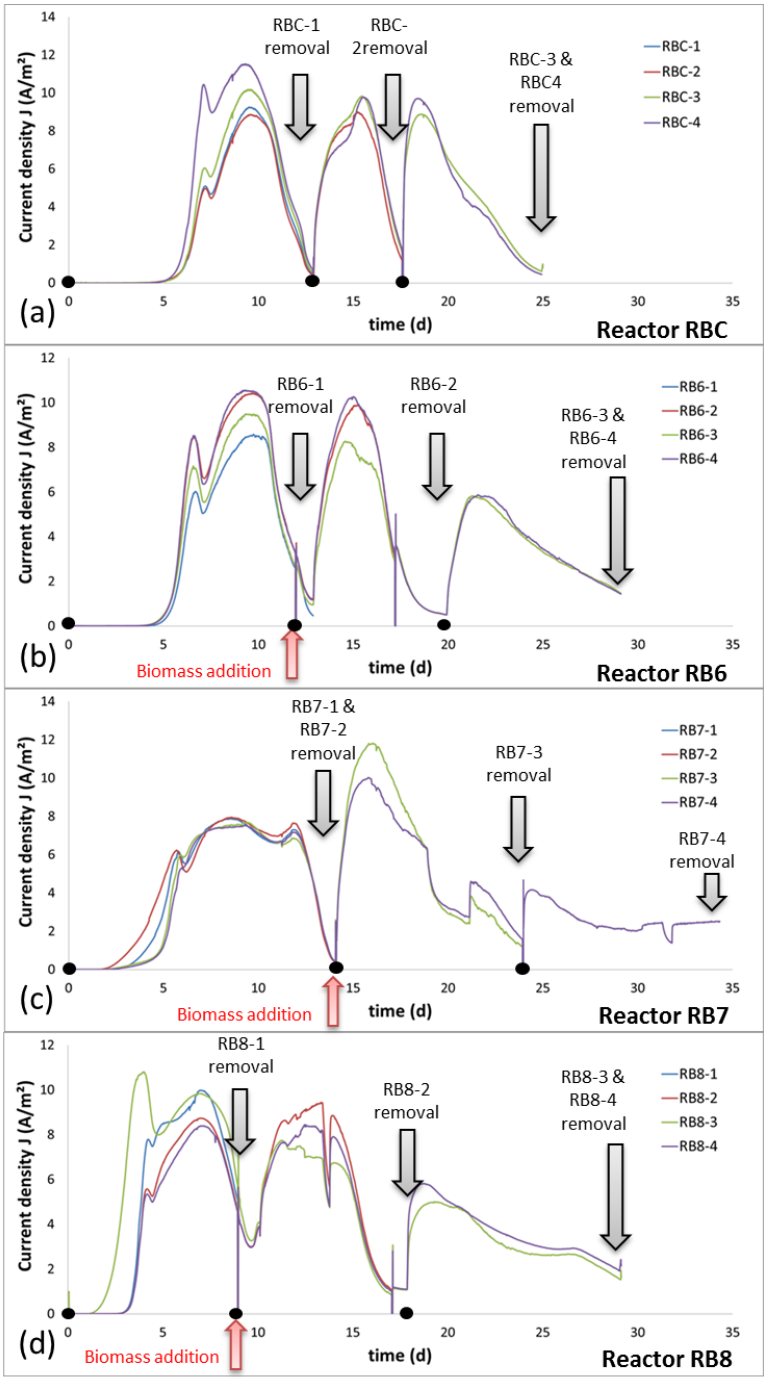


Figure V-5 : Chronoamperometry in the different systems:

Control reactor labeled RBC and biomass contaminated reactors with microbial communities harvested from batch fermenters performed at: (b) pH 6 for reactor RB6, (c) pH 7 for reactor RB7 and (d) pH 8 for reactor RB8. Black arrows present the removal of biofilms and red arrow present the biomass injection time. Biomass was injected after the first batch cycle. In the case of pH 7 batch contamination, 2 biofilms were collected first, due to technical constraint. On each graph, ● presents acetate injection.

Tableau V-1: Coulombic efficiencies (%) and maximum current densities (A/m²) obtained from biofilms in the different reactors for each acetate-feeding cycle.

RBC is the control reactor without biomass addition and RB6, RB7 and RB8 are the contaminated reactors with biomass originated from batch fermenters respectively conducted under pH 6, pH 7 and pH 8. Cycles are labeled 1, 2 and 3 for successive batch cycles.

Reactor	C_E (%) Jmax (A/m²)	Cycle 1	Cycle 2	Cycle 3
RBC	C_E	108%	88%	101%
	J_{max}	9.3±0,1	9.4±0,5	9.2±0,6
RB6	C_E	123%	93%	108%
	J_{max}	9.8±1,0	9.4±1,2	5.7±0,1
RB7	C_E	154%	168%	156%
	J_{max}	7.7±0,2	10.8±1,3	4.1
RB8	C_E	105%	132%	105%
	J_{max}	9.6±0,8	8.4±0,9	5.4±0,5

V.2.3.2.2. *Impact of exogenous fermentative bacteria on biofilm communities*

The 16S rRNA gene based CE-SSCP profiles of bacterial communities in reactors RBC, RB6, RB7 and RB8 are presented in Figure V-6. These profiles present very simple community structures with low Simpson diversity indexes of 0.85±0.05 in comparison to the inoculum diversity index of 0.99. A strong evidence of bacterial selection emerges with only one or two main peaks as dominant bacteria for each biofilm bacterial community. No specific trend of the Simpson diversity indexes among cycles was shown. These results show that there was no impact of exogenous bacteria originating from dark fermentation batch tests on biofilm bacterial community structures.

Since an increase of the biofilm thickness was observed among cycles in reactors RB6, RB7 and RB8, we assessed the quantity of bacteria per surface unit on electrodes with quantitative PCR. Analyses operated on all biofilm revealed no difference in quantity of bacteria among cycles and between reactors (data not shown). Again, this suggests that the increase of thickness observed for RB6, RB7 and RB8 could be due to an EPS production in order to protect settled bacteria (Burmølle et al., 2006) more than a bacterial growth.

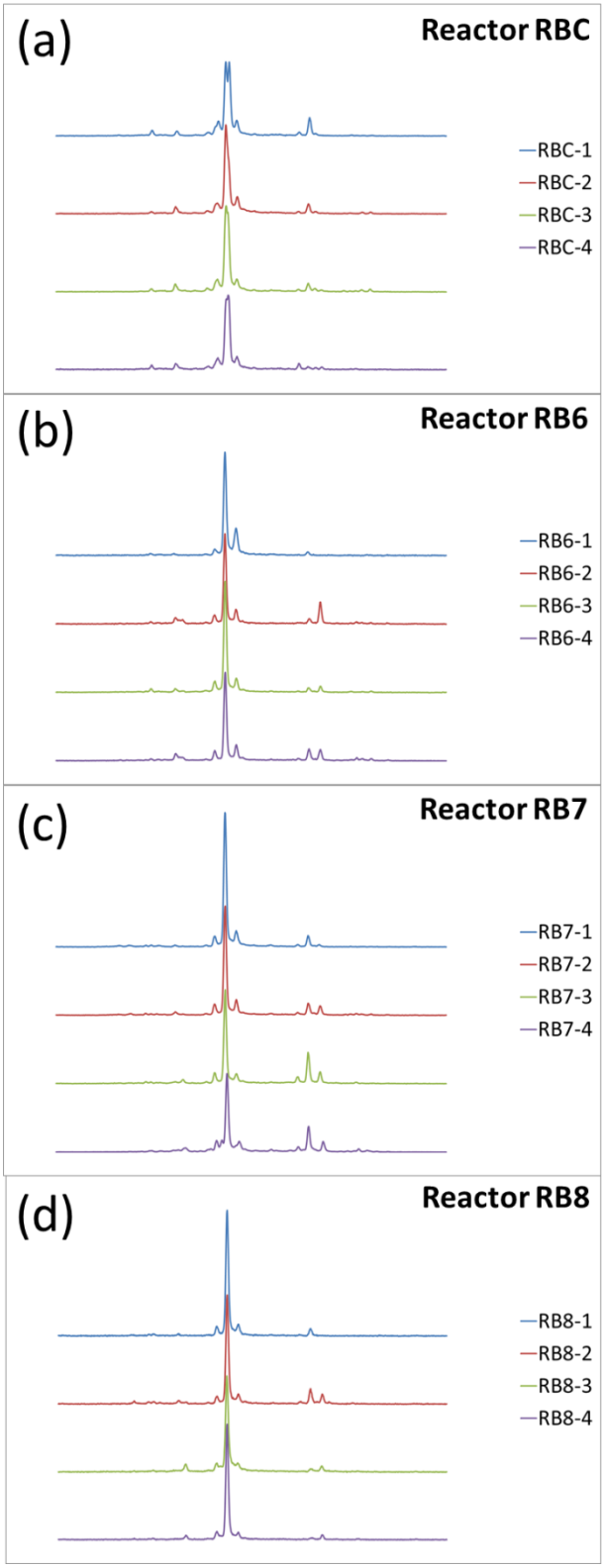


Figure V-6 : CE-SSCP profiles of biomass based on 16S rRNA gene fragments retrieved from biofilms in the 4 different reactors:

(a) Control and (b), (c) and (d) biomass contaminated reactors with microbial community harvested from batch fermenters performed at: (b) pH 6, (c) pH 7 and (d) pH 8. The profiles correspond to sampling times indicated on Figure V-5. X and Y axes of each peak represent respectively the relative peak electrophoresis migration distance and the relative peak intensity (Arbitrary units).

V.2.3.2.3. *Bacterial Identification results*

V.2.3.2.3.1 *Fermentative biomass*

16S rDNA Pyrosequencing of pH 8 dark fermentative batch culture led to the identification of the main bacteria selected during fermentation process. These bacteria (percentage up to 1%) were related to *Psychrobacter psychrophilus* (64.1%), *Psychrobacter* sp. (21.2%), *Nesiotobacter* sp. (7.8%) and *Arthrobacter protophormiae* (3.4%). Despite *Psychrobacter* species have been described as aerobic and mesophilic bacteria, few studies showed that some of these bacteria can grow in fermenting environments (Yoon, 2003; Yoon, Lee, Kang, et al., 2005; Yoon, Lee, Yeo, et al., 2005; Li et al., 2013), which is concomitant with the selection operated of these main bacteria in dark fermentation batch reactors.

V.2.3.2.3.2 *Biofilms*

Tableau V-2 presents the percentage of species representing 1% or more of the bacterial community developed in biofilms of the first bioreactor contaminated with pH 8 batch fermenters biomass (RB8) and the electroactive biofilms developed in the uncontaminated bioreactor (RBC). These results had been obtained by 16S rDNA pyrosequencing. These results are in accordance with bacterial community structure fingerprints (Figure V-6) where no trend in microbial selection on the electroactive biofilm for reactor RB8 was observed. What is brought out here is that the maximum current density can be such high with an electroactive biofilm composed of more than 90% of *G. subterraneus* as with an electroactive biofilm composed of 65% *G. subterraneus*, 16% *Desulfuromonas* sp. and 16% *Malonomonas rubra*.

Desulfuromonas spp. has been widely studied in the literature as an ARB (Bond et al., 2002; Bond and Lovley, 2003; Holmes, Bond, O'Neil, et al., 2004; Logan and Regan, 2006; Nevin et al., 2009; Dumas et al., 2008b; Nevin et al., 2008; Call et al., 2009; Wei et al., 2010; Marsili et al., 2010; Qu et al., 2012; Miceli et al., 2012; Nercessian et al., 2012). In contrast, only one recent study found bacteria genetically related to *G. subterraneus* as a dominant ARB in mixed culture, i.e. in the microbial population of electroactive microbial biofilms enriched from shoreline and mangrove sediments (Miceli et al., 2012). *Geoalkalibacter* was dominant in highly enriched biofilms producing significant current densities (4.2 to 8.9

A/m²). Additionally, *G. subterraneus* anodic electron transfer was independently evidenced using a pure culture by two different research groups (Carmona-Martínez 2013; Badalamenti 2013). In those studies, *G. subterraneus* provided high current densities with respectively 5.06±0.15 A/m² and 5.0 A/m².

Tableau V-2 : Taxonomic classification of bacterial taxonomic distribution (in %) according to biofilms retrieved from N-stat control reactor and from N-Stat contaminated reactors with fermentative biomass grown at pH 8.

Names correspond to the closest phylogenetical known sequence depending on percentage of identity (>97% for species identification). Relative abundance was defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample. Samples making up less than 1% of total composition were grouped in "others".

Name	RB8-1	RB8-2	RB8-3	RB8-4	RBC-1	RBC-2	RBC-3	RBC-4
	Reactor RB8				Reactor RBC			
Geoalkalibacter subterraneus	**96,1**	**89**	**94**	**93,7**	**65,8**	**76,9**	**72**	**59,4**
***Desulfuromonas* sp.**	3,2	7,9	1,5	1,1	16,4	7,4	7,9	2
Desulfuromonas acetoxidans	0	0	0	0	0,4	0	0	0
Malonomonas rubra	0	0	0,1	0	15,6	13,9	19	38,2
Clostridium halophilum	0,4	1,8	1,1	1,4	0,1	0,1	0,0	0,0
Others	0,3	1,3	3,3	3,8	1,7	1,7	1,1	0,4

Malonomonas rubra was interestingly described as a microbe phylogenetically closely related to *Desulfuromonas acetoxidans* (about 92% similarity between 16S rRNA sequences (Kolb et al., 1998)). This bacterium was shown to be particularly interesting as it produces c-type cytochrome similar to those of sulphur and sulphate reducers (Kolb et al., 1998). The presence of *Malonomonas rubra* in RBC biofilms can be explained by its ability to an external electron transfer which is similar to anodic respiration (Roden and Lovley, 1993; Wang et al., 2010).

The maintenance of RB8 biofilms among cycles and the absence of changes in bacterial community structure suggested that synergetic relations could permit to protect this structure against external factors. The involvement of exogenous bacteria in syntrophic

relation has already been shown, based on substrate consumption (Parameswaran et al., 2009; Kiely, Regan, et al., 2011). Moreover, the rather high current densities reached compared to the literature on graphite plate electrodes were similar to previous studies (Erable et al., 2009; Miceli et al., 2012) supported the idea that this mixed culture could develop a strong resistance to external perturbations. Indeed, there was no settlement of *Psychrobacter psychrophilus*, selected in pH 8 dark fermenters as main bacteria, neither of other minor population of the exogenous community.

V.2.4. CONCLUSION

The objectives of this research were to determine if the electron transfer performances and the bacterial community structure of the ARB biofilm could be affected by the exogenous bacteria originated from several dark fermentation pH conditions. Our results show that the electroactive biofilm originated from sediment presents a robust biofilm showing high current densities and a robust bacterial community structure. The input of exogenous bacteria from batch dark fermenters has no impacts on this strong biofilm bacterial structure. Nevertheless, these exogenous species addition affects the current density generated by the biofilm likely owing to EPS production and methane production occurring in such batch conditions.