SELECTION ET IDENTIFICATION DE MICROORGANISMES ELECTRO-ACTIFS

IV.1.1. AVANT-PROPOS

Les microorganismes électroactifs impliqués dans les systèmes bioélectrochimiques (BES) tels que les piles à combustibles microbiennes (MFC – « Microbial Fuel Cells ») ou les cellules d'électrolyse microbiennes (MEC – « Microbial Electrolysis Cells ») sont actuellement très étudiés et l'optimisation des procédés comme la recherche de consortia électroactif plus performant est d'un intérêt majeur pour les futures applications industrielles (Logan, 2009; Lovley, 2012). Dans le cadre de cette thèse, un consortia type a été recherché pour assurer le couplage de la fermentation et de l'électrolyse microbienne.

Dans les systèmes bioélectrochimiques, le transfert de charges joue un rôle très important et l'influence de la salinité dans le compartiment anodique fait l'objet de récentes études (Liu, Cheng, et al., 2005b; Lefebvre et al., 2012; Rousseau et al., 2013). Liu et al., (2005a) ont observé qu'une augmentation de 1,7 à 6,8 g_{NaCl}/L induisait une augmentation de 85% de la densité de puissance en utilisant des eaux usées domestiques comme inoculum. Plus récemment, Lefebvre et al. (2012) ont signalé une augmentation des densités de puissance en pile à combustibles microbienne en utilisant des concentrations plus élevées en NaCl de 0,0 à 20,0 g_{NaCl}/L. Dans ce cas, une salinité plus importante s'avérait être préjudiciable pour la performance globale des BES, signifiant que l'activité electroactive des biofilms obtenus à partir d'eaux usées domestiques pouvait être fortement diminuée pour des salinités relativement faibles (de l'ordre de 20-30 g_{NaCl}/L). L'amélioration des performances a été donc très limitée en termes de gamme de salinité au cours de ces études, pour lesquelles les inocula utilisés pour la sélection d'espèces électroactives sur anode étaient essentiellement d'origine non salée. Récemment, Rousseau et al. (2013) ont montré une influence positive de la salinité sur les performances électroactives du biofilm pour des salinités de l'ordre de 20 g_{NaCl}/L avec des biofilms formés à partir de sédiments issus d'un environnement salin.

De plus, la culture en milieu salin de biofilms électroactifs permettrait le traitement d'effluents salins encore peu traités tels que les effluents d'industries de la pêche, du pétrole et du cuir ou certaines eaux usées rejetées par les usines de traitement d'eau potable. Ces effluents difficiles à traiter par les procédés biologiques conventionnels présentent un risque

important de salinisation des sols et des eaux de surface et souterraines (Lefebvre et al., 2010, 2012; Pierra et al., 2013).

Dans un premier temps, l'objectif de ce travail a été d'identifier de nouvelles sources de consortia bactériens électroactifs. Pour cela, la sélection de biofilms électroactifs a été réalisée dans des conditions salines. Ainsi, cette étape a été testée en milieu salin (30 g_{NaCl}/L) avec une salinité proche de celle constatée dans la majeure partie des mers et océans, qui devraient ainsi représenter une salinité optimale pour la croissance d'une plus grande variété d'organismes.

De plus, la sélection de bactéries électroactives à partir de ces sédiments salins produirait un consortium efficace pour travailler dans des BES à des salinités plus élevées que celles habituellement appliquées. Ainsi des inocula anaérobies en incluant des sédiments issus de milieux salins.

Les biofilms ont été formés en utilisant un système a 3 électrodes avec une électrode de travail en graphite polarisée à +0.2V/Ag/AgCl. Différentes sources d'inocula issus de milieux anaérobies ont été utilisées à différents pH afin de sélectionner des populations électroactives comme décrit dans le *Tableau IV-1* ci-dessous (sédiments d'égout de salins de Gruissan mais aussi vinasses, compost, sol). Les pH choisis ici se rapprochaient des pH des milieux de prélèvement des inocula. L'électrolyte utilisé (milieu réactionnel) était composé d'oligo-éléments, d'eau de mer ou d'une solution de NaCl à 30 g_{NaCl}/L. Les plus performants, en termes de production de courant, sont les sédiments de l'étang de Gruissan (4.2 A/m², dans l'eau de mer à pH 7) et un échantillon de sol (2.0 A/m² dans l'eau de mer à pH 6).

Tableau IV-1 : Recherche d'inocula pour la formation d'un biofilm électroactif dans un système a 3 électrodes avec une électrode de travail en graphite polarisée à +0.2V/AgAgCl.

Inoculum	pH	temps de latence (j)	Jmax (A/m²)
Fermenteur	5,5	-	0
Curet	5,5	-	0
Vinasses	6	-	0
Compost	6	-	0
Sol	6	9	2
Mélange (vinasses + compost + sol)	6	21	0,1
Sédiments de l'étang de Gruissan	7	2	4,2

Ces premières expériences ont permis d'obtenir de bonnes densités de courants notamment avec l'inoculum de sol (2 A/m²) et celui des sédiments de l'étang de Gruissan (4.2 A/m²) à des pH respectifs de 6 et 7. Les faibles pH semblent empêcher la formation d'un biofilm électroactif. Ces résultats sont cohérents avec la littérature dans laquelle la majorité biofilms électroactifs sont formés à partir de sols et de sédiments d'eaux douces ou salines (Lovley, 2006b). Dans une étude précédente effectuée dans des conditions salines (20 g_{NaCl}/L) , Erable et al. (2009) ont montré une production efficace de courant (2,5 A/m²) en utilisant un biofilm naturel marin fixé sur une électrode en graphite polarisée à -0,1 V/ESC et alimenté avec de l'acétate à 10 mM. L'intérêt d'utiliser des sédiments salins comme source de micro-organismes électroactifs est ici vérifié avec l'obtention d'une densité de courant de 4.2 A/m² pour le biofilm obtenu à partir des sédiments de l'étang de Gruissan. Plus récemment, Rousseau et al. (2013) ont observé, en utilisant un inoculum issu d'un milieu salin, une augmentation de la production de densité de courant dans des conditions salines dans un système à potentiel contrôlé alimenté avec 40 mM d'acétate. Une densité de courant de 85 A/m² a même été obtenue à 45 g_{NaCl}/L sur un feutre de graphite. Une diminution de la production de densité de courant a ensuite été observée à 60 g_{NaCl}/L mais elle restait encore élevée (30 A/m²). Une caractérisation des communautés microbiennes de ce type de biofilms développés en conditions salines apporterait un éclairage nouveau et sans doute nécessaire à la compréhension des résultats observés en termes de densités de courant.

Dans un deuxième temps, un inoculum type à utiliser dans le cadre du couplage de la fermentation et de l'électrolyse microbienne a été défini et la capacité d'adaptation des consortia aux conditions de fonctionnement en MEC a été évaluée sur les biofilms ainsi obtenus. Suite aux tests préliminaires, il a donc ensuite été décidé de sélectionner des bactéries électroactives à partir de 2 types d'inoculum : les sédiments de l'étang de Gruissan et les sédiments des salins de Gruissan (qui ont été testés à pH 7). L'originalité de cette étude a été d'utiliser des sédiments salins, déjà adaptés à une concentration en sel importante, dans un milieu de culture salin. Les biofilms ont été étudiés en termes de performances électroactives (densité de courant, rendement faradique, temps de latence), mais aussi en termes de composition des communautés microbiennes.

Ainsi, dans l'étude présentée dans le premier article en IV.1.2, des biofilms électroactifs ont été sélectionnées sur anodes dans des conditions salines sous différentes conditions de pH et de donneur d'électrons (avec de l'acétate et du butyrate comme substrat) (phase 1). Dans le cadre du couplage de la fermentation et de l'électrolyse microbienne, les expériences en phase 1 ont été réalisées à pH neutre ou acide, en présence d'acétate ou d'un mélange d'acétate et de butyrate qui correspondent respectivement aux conditions standards utilisées dans les systèmes bioélectrochimiques (Logan, 2012) et à la composition de la sortie d'un fermenteur de production de bioH_2 (Guo et al., 2010). Deux sédiments salins différents ont ensuite été testés dans une deuxième série d'expérience (phase 2) pour inoculer les réacteurs électrochimiques à potentiel contrôlé dans les conditions ayant donné les meilleures performances d'électroactivité en phase 1. Les communautés microbiennes des biofilms ont ensuite été caractérisées par PCR-CE-SSCP et 454 pyroséquençage.

IV.1.2. CROISSANCE DE BIOFILMS ELECTROACTIFS ET PRODUCTION DE COURANT ELEVEES DANS DES CONDITIONS SALINES, SELECTION DE GEOALKALIBACTER SUBTERRANEUS

Electrochemical growth of high current producing biofilms under saline conditions drives the efficient selection of *Geoalkalibacter subterraneus*

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Running headline: Selection of *Glk. subterraneus* in electroactive biofilms

Abstract

Aims: The objective of this work was to assess the electrochemical and microbiological characterization of anodic biofilms derived from saline sediments under well potentiostatically controlled conditions in a bioelectrochemical system (BES) operated in saline conditions (35 g_{NaCl} l^{-1}).

Methods and Results: The performance of anodic biofilms was studied in terms of biofilm formation lag-phase, maximum current density production and coulombic efficiency. Additionally, microbial communities were characterized by using PCR-SSCP and 454 pyrosequencing. Potentiostically controlled reactors were used for the growth of electroactive biofilms fed with only acetate or a mixture of acetate and butyrate at two pH values: 7.0 or 5.5. Two inocula were tested: sediments from a salt lake and from a salt-producing platform. High current densities, up to 8.5 A m^{-2}, were obtained in graphite planar electrodes as a consequence of a strong electrochemical driven selection wherein only one or two species of ARB were selected as main dominant bacteria in anodic biofilms.

Conclusions: Efficient electroactive biofilms could be grown under saline conditions with the selection of *Geoalkalibacter subterraneus* and/or *Desulfuromonas* spp. as dominant species.

Significance and Impact of the Study: This study reveals the high selection in electrochemically driven biofilms of anode-respiring bacteria from saline inocula and under saline conditions. The enrichment of such halophilic biofilms provides an alternative for the treatment of saline wastewaters, usually limited by technical or economic constraints.

Keywords Bioelectrochemical systems, Capillary-Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP), 454 Pyrosequencing, *Geoalkalibacter subterraneus, Desulfuromonas spp*., Saline wastewater

IV.1.2.1. INTRODUCTION

Microorganisms embedded in electroactive biofilms are able to convert organic matter into electrical current in bioelectrochemical systems (BES) (Logan et Regan 2006; Arends et Verstraete 2012; Lovley 2012) for the production of electricity in microbial fuel cells (MFCs) (Rabaey et Verstraete 2005; Logan et Regan 2006; Rozendal *et al.* 2006) or the production of hydrogen in microbial electrolysis cells (MECs).

Several anode respiring bacteria (ARB) embedded in electroactive biofilms are able to directly transfer electrons to/from electrode materials (Patil et al., 2012) and the study of their electrode interactions has allowed a progressively increase of the current densities achieved in BESs up to 390 A m^{-2} (Chen *et al.* 2012).

On the other side, it has been extensively found that ARB are related to α, β, γ, δ-*Proteobacteria*, *Firmicutes*, *Acidobacteria* and *Actinobacteria* (Chaudhuri et Lovley 2003; Holmes *et al.* 2004; Zuo *et al.* 2008; Liu *et al.* 2010). While wastewater is commonly the source of microorganisms in BESs for the development of electroactive biofilms, its applications are consequently limited to the treatment of domestic wastewater (Lefebvre *et al.* 2010). Hence, the selection of halophilic ARB embedded in microbial biofilms is an essential prerequisite for the treatment of saline wastewaters (SWWs) that represent a high risk of soil, surface and groundwater salinization (Lefebvre *et al.* 2010, 2012; Pierra *et al.* 2013).

On the other hand, although it is known that saline conditions could increase the conductivity of electrolyte solutions and facilitate the proton transport with the consequent increment of the overall BES performances (Lefebvre *et al.* 2012), this has been rarely tested. Liu (2005a) observed that an increment from 1.7 to 6.8 g_{NaCl} l^{-1} induced an 85% increase in power densities (Liu *et al.* 2005a) in domestic wastewater based BESs. More recently, Lefebvre (2012) reported an increase of power densities by progressively using higher concentrations of NaCl from 0.0 up to 20.0 g_{NaCl} l^{-1}. Higher salinity concentration

proved to be detrimental for the overall BES performance, meaning that ARB enriched from domestic wastewater were affected at relatively low salinities (Lefebvre *et al.* 2012).

Interestingly, previous studies assessing the impact of salinity on bioelectrochemical activity of ARB were conducted by using domestic wastewater as inoculum (Lefebvre *et al.* 2012; Liu *et al.* 2005a), although saline sediments can be a better source of suitable halophilic ARB for such studies in BES systems (Bond *et al.* 2002; Lovley 2006; Erable *et al.* 2009; Miceli *et al.* 2012). The selection of ARB from such saline sediments for the formation of electroactive biofilms would produce an efficient consortium to work in BESs under high salinity concentrations that are not usually tested.

In a previous study performed under saline conditions (20 g_{NaCl} L^{-1}), Erable (2009) showed an efficient current production of 2.5 A m^{-2} using a natural marine biofilm on a graphite electrode polarized at -0.1 V/SCE and fed with 10mM acetate. Recently, Rousseau (2013) observed an increase of current density production under saline conditions in a potentiostically controlled system fed with 40 mM acetate. A current density of 85 A m^{-2} was obtained at 45 g_{NaCl} l^{-1} on a porous carbon felt anode. A decrease of current production was then observed at 60 g_{NaCl} l^{-1} but the performance was still significant (30 A m^{-2}). However, the lack of information on the microbial communities in such a saline biofilm hinders the overall understanding of the produced current densities.

In the present work, the enrichment of electroactive biofilms under saline conditions under different conditions of pH and electron donor (Phase 1) is reported. Since this work is part of a project aiming at feeding BES with dead-end metabolites from dark fermentation effluents, the pH tested were neutral or acidic pH for conditions in BES (Logan, 2012) and dark fermentation (Guo *et al.* 2010) respectively. Acetate alone or a mixture of acetate and butyrate were used as electron donors. Additionally, two different saline sediments were tested to inoculate potentiostically controlled electrochemical reactors in the conditions showing the best performance in phase 1 in terms of current production (Phase 2). Biofilms were then extensively analyzed to describe the high selection that occurred in the anodic biofilm. This selection was quantitatively and easily characterized by the use of PCR-SSCP and 454 pyrosequencing. An approach used for the first time for the characterization of highly enriched ARB in anodic biofilms (Harnisch and Rabaey 2012).

IV.1.2.2. MATERIAL AND METHODS

IV.1.2.2.1. *Origin of the Inoculum*

As briefly explained above, the inocula used here were sampled from two different locations: Inoculum 1, sediments collected at a salt lake beach and Inoculum 2, seed sediments from a lagoon collecting wastewaters from a salt factory (Salins de Saint Martin, Gruissan, France). Both inocula presented similar physicochemical properties, i.e., pH of 7.8±0.2; 36.1±3.5 $g_{VS}\, g_{sediments}^{-1}$ and a value of conductivity of 93.6±12.1 mS cm^{-1}.

IV.1.2.2.2. *Experimental set-up: potentiostically controlled electrochemical reactors*

Experiments were carried out using electrochemical reactors under potentiostatic control (VSP Bio-Logic SA) monitored with a computer (EC Laboratory v.10.1 software, Bio Logic SA). The set-up, based on a previously described arrangement (Ketep *et al.*, 2013), consisted of a working electrode (graphite), a reference electrode (Ag/AgCl) and a counter electrode (platinum grid). The anodes were geometrically well defined graphite planar working electrodes with the following dimensions: 2.5x2.5x0.2 cm (Goodfellow). To ensure the electrical connection, 2 mm diameter and 12 cm long titanium rod (Goodfellow) were used. The working electrodes were cleaned before use using a P800 sandpaper and rinsed with 99% purity ethanol. Cathode or counter electrodes were Platinium Iridium grid (90%/10%) (Heraeus) cleaned by heating in a blue flame. The potentials of working electrodes were controlled using an Ag/AgCl electrode (potential +0.197 vs. SHE).

Inocula were added into the culture media (10% v/v for a final working volume of 400mL) containing 50mM of MES buffer and mineral solution (Rafrafi *et al.* 2013) (0.5 g l^{-1} K_2HPO_4, 2 g l^{-1} NH_4Cl, 0.2 g l^{-1} Yeast Extract, 55 mg l^{-1} $MgCl_2$, $6H_2O$, 7 g l^{-1} $FeSO_4(NH_4)2SO_46H_2O$, 1mg l^{-1} $ZnCl_2$, $2H_2O$, 1.2 mg l^{-1} $MnCl_2$, $4H_2O$, 0.4 mg l^{-1} $CuSO_4$, $5H_2O$, 1.3 mg l^{-1} $CoSO_4$, $7H_2O$, 0.1 mg l^{-1} BO_3H_3, 1 mg l^{-1} $Mo_7O_{24}(NH_4)_6$, $4H_2O$, 0.05 mg l^{-1} $NiCl_2$, 6 H_2O, 0.01 mg l^{-1} Na_2SeO_3, 5 H_2O, 60 mg l^{-1} $CaCl_2$, 2 H_2O).

Reactors were fed with acetate (10 mmol l^{-1}) or a mixture of acetate (5 mmol l^{-1}) and butyrate (5mM) as described in Tableau IV-2. Deionized water (reactors B7, B8, B9) or sea water (reactors B1, B2, B3, B4, B5, B6) were used in order to get a final salinity equivalent to 30 $g_{NaCl}\, L^{-1}$. The initial pH was adjusted to 5.5 or 7.0 using NaOH (1 mol l^{-1}).

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 Ag/AgCl during chronoamperometry (CA).

CA maximum current densities j_{max} (A m^{-2}) of mature microbial biofilms were calculated considering the total immersed electrode surface area since electroactive biofilms covered both sides of the electrode with the same microbial community profile (CE-SSCP patterns). Coulombic efficiencies (CE) were calculated for each experiment according to (Call et al. 2009).

IV.1.2.2.3. *Chemical analyses*

Volatile Fatty Acids (VFA) consumption (*i.e.* acetic (C2) and butyric (C4) acids) was determined with a gas chromatograph (GC-3900 Varian) equipped with a flame ionization detector. Sample analysis was performed using an auto sampler. A syringe automatically takes 0.5µl of the mix and places it in the injector, heated at 250°C. The column used is an ELITE FFAP, 15000x0.53 mm (Perkin Elmer). The temperature is maintained at 120°C. The carrier gas is nitrogen (N_2) and the flame ionization detector gas is hydrogen (H_2).

IV.1.2.2.4. *DNA extraction, PCR amplification and CE-SSCP fingerprinting*

All DNA from biofilms obtained was extracted and the microbial community on biofilms and inoculum 2 was 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).

On DNA samples selected from CE-SSCP profiles, pyrosequencing was performed using a 454 protocol for bacterial identification (Research and Testing Laboratory (Lubbock, USA)). Sequences of most abundant bacteria found in each biofilm were deposited in the NCBI genbank database under the following accession numbers for the main abundant bacteria selected in anodic biofilms (listed as “sequence number” for “biofilm designation”): KF573509 for B1; KF573510 for B2; KF573511 for B3; KF573512 and KF573513 for B4; KF573514 for B5; KF573515 for B6; KF573516 for B7; KF573517 and KF573518 for B8 and KF573519 for B9.

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).

IV.1.2.3. RESULTS

IV.1.2.3.1. *Phase 1: enrichment of electroactive biofilms under saline conditions*

Representative CA curves are presented in Figure IV-1 to exemplary show how lag phase and maximum current density were calculated. Additionally, it is shown the time at which biofilm sampling for microbial community analysis took place.

By using the same inoculum source (Inoculum 1), two pH values and two different electron donors (see Phase 1 in Tableau IV-2) j_{max} ranged from 1.9 to 4.2 A m^{-2}, for reactors B1 to B4, respectively. CEs varied between 11 % (B1) to 70 % (B4) for respective (mixture of acetate and butyrate fed, pH 7) and (acetate fed, pH 7) conditions. The low CE achieved could be an indication of the use of the supplied substrate for biosynthesis instead of its direct conversion into current (Logan et Regan 2006). Among those 4 reactors, the highest j_{max} (4.2 A m^{-2}) was obtained at neutral pH with acetate as sole carbon source, with the highest CE (70 %) and shortest lag phase (1.46 days). Hence, further experiments were performed with the best conditions found in Phase 1 (see below).

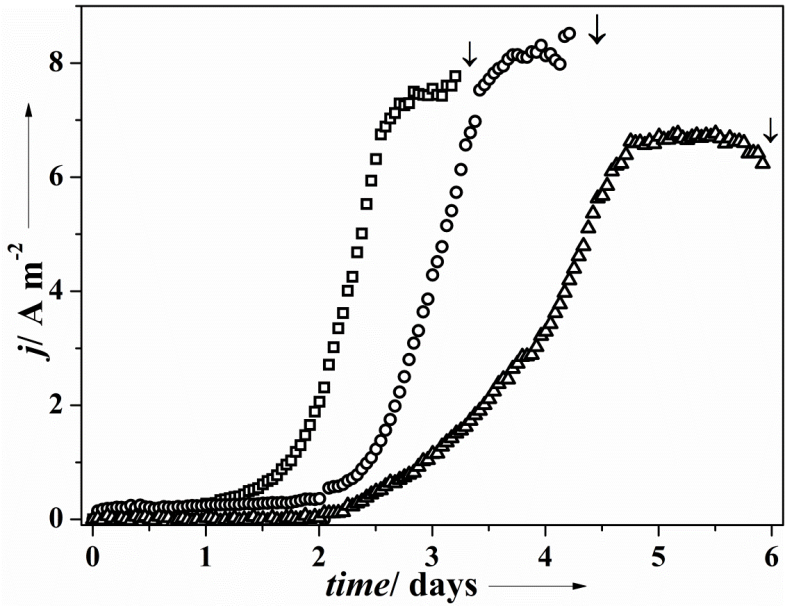

Figure IV-1 : Representative chronoamperometric batch cycles of electrochemically derived biofilms B7(Δ), B8(□) & B9(○).

Experiments realized under saline conditions (30 g l-1 NaCl) and pH 7.0 at graphite planar electrodes (15 cm2). Applied potential: +200 mV vs. Ag/AgCl. Inoculum site 2 and acetate as carbon source. Arrows indicate anodic biofilm collected for microbial analysis. Solid and dashed horizontal lines indicate lag and exponential growth phases, respectively.

IV.1.2.3.2. *Phase 2: enhancement of current density (j_{max}) and Coulombic efficiency (CE)*

Five additional potentiostically controlled electrochemical reactors were inoculated with sediments from a salt lake or from an industrial salt-producing platform (see Phase 2 in Tableau IV-2). In average, for reactors fed with acetate at pH 7, j_{max} were 7.6±0.9 A m^{-2} and 5.0±1.1 A m^{-2} and CEs were 63.3±12.3 % and 75.7±4.9 % with reactors inoculated with sediments from the salt factory (inoculum site 2) and the salt lake (inoculum site 1), respectively. It can be clearly observed from the overall performance of electrochemical reactors in terms of j_{max} and CE that experiments performed in phase 2 outperformed those of phase 1, mainly due to the use of well controlled physicochemical conditions (such as pH, temperature and anodic potential) that allow the enrichment of ARB from saline sediments. On the other side, the lag-phase could be clearly decreased from phase 1 to 2 in less than 1 day thanks to the experience gathered in phase 1 and due to the use of well controlled experimental conditions as outlined before.

Tableau IV-2 : Performance of electrochemically derived biofilms grown under saline conditions

Experimental conditions*	Lag phase/d	j_{max}/ A m^{-2}	CE/ %	Main dominant species identified in the biofilm†
Phase I, effect of pH, substrate and type of salinity on biofilm formation and performance				
B1, pH 7.0, In 1, Ac-Bu, Sea water	2.94	1.9	11	*Desulfuromonas acetoxidans* (86.09%)
B2, pH 5.5, In 1, Ac-Bu, Sea water	9.36	2.2	21	*Desulfuromonas acetoxidans* (98.29%)
B3, pH 5.5, In 1, Ac, Sea water	8.71	2.7	12	*Desulfuromonas acetoxidans* (99.16%)
B4, pH 7.0, In 1, Ac, Sea water	1.46	4.2	70	*Geoalkalibacter subterraneus* (39.32%) *Desulfuromonas acetoxidans* (46.72%)
Phase II, effect of inoculum on biofilm formation and performance				
B5, pH 7.0, In 1, Ac, Sea water	2.20	4.5	80	*Geoalkalibacter subterraneus* (95.43%)
B6, pH 7.0, In 1, Ac, Sea water	1.77	6.3	78	*Geoalkalibacter subterraneus* (98.59%)
B7, pH 7.0, In 2, Ac, NaCl	1.86	6.7	60	*Geoalkalibacter subterraneus* (72.86%)
B8, pH 7.0, In 2, Ac, NaCl	0.27	7.7	53	*Geoalkalibacter subterraneus* (20.08%) *Desulfuromonas* spp. (58.18%)
B9, pH 7.0, In 2, Ac, NaCl	1.90	8.5	77	*Geoalkalibacter subterraneus* (91.24%)

*Construction of experimental unit name. B1-9: biofilm number, pH: 7.0 or 5.5, Inoculum site: 1 or 2, Substrate: acetate (Ac) or butyrate (Bu) and type of salinity adjustment: sea water or NaCl; †Names in italics correspond to the closest phylogenetical known sequence depending on the percentage of identity. Numbers in parenthesis represent the relative abundance values for each species obtained from 454 pyrosequencing analysis.

IV.1.2.3.3. *Structure and composition of anodic bacterial communities*

16S rRNA gene-based CE-SSCP fingerprint profiles of the biofilms are presented in Figure IV-2. Simpson diversity index was also calculated to describe the level of species diversity in an environment (Braun *et al.* 2011; Simpson 1949).

These community profiles exhibited simple structures with an average Simpson index of 0.86±0.06. In comparison, the inoculum was more diverse with a Simpson diversity index of 0.98±0.002. Only one or two main peaks were present as main dominant bacteria for each anodic biofilm. This confirms the high selection of ARB occurring in anodic biofilms of well potentiostically controlled BESs; a phenomenon described in previous studies (Bond *et al.* 2002; Freguia *et al.* 2010; Harnisch *et al.* 2011). Analysis of 16S ribosomal DNA genes by 454 pyrosequencing showed a pronounced dominance of microorganisms from the delta subgroup of *Proteobacteria* colonizing the anodes. From 85.4% to 97.0% of the 16S DNA sequences from microorganisms colonizing the anodes were related to this subgroup. Furthermore, 83.5% to 98.6% of the sequences relative abundance increase was due to few clusters of bacteria affiliated to *Desulfuromonadaceae* spp. (*Desulfuromonas* spp.) and to *Geobacteraceae* spp. (*Geoalkalibacter subterraneus, Geoalkalibacter* spp and *Geobacteraceae* (unknown genus) (see Tableau IV-2).

Finally, most abundant species found in all electroactive biofilms were closely related and were up to 97% similar to *Desulfuromonas* spp and *Geoalkalibacter subterraneus* in both sediment-inoculated reactors. The later ARB, only reported in a recent BESs study Miceli (2012) showing *Geoalkalibacter* dominated biofilms derived from environmental anaerobic samples. Even after the high selection found in the experiments presented here, no specific trend in terms of ratio of main bacteria could be noticed in the selection of bacteria among conditions tested.

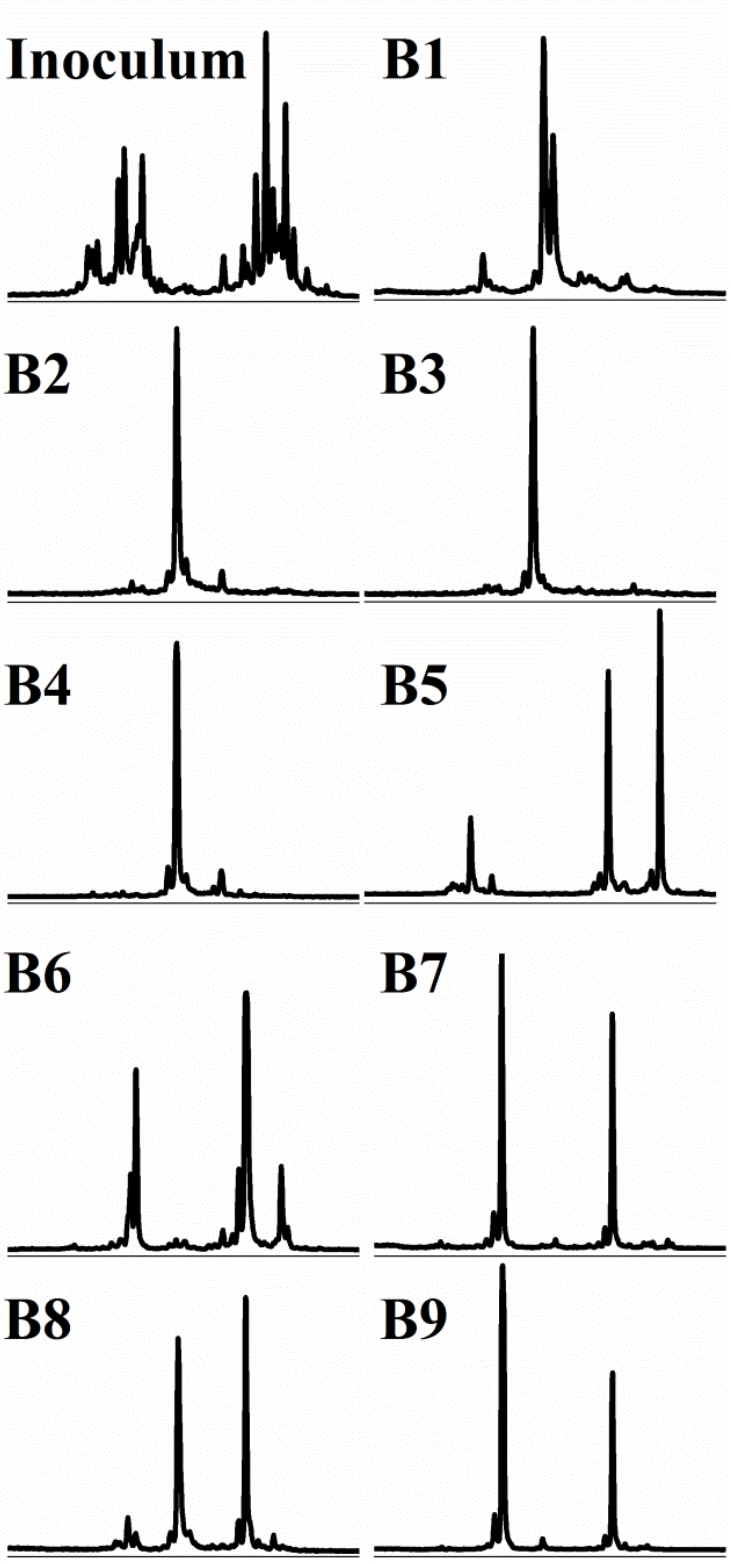

*Figure IV-2 : CE-SSCP profiles based on 16S rRNA gene fragments retrieved from electroactive biofilms R1 to R9 grown in different operating conditions (see*Tableau IV-1*).*

The X and Y axes of each peak represent respectively the relative peak electrophoresis migration distance and the relative peak intensity (Arbitrary Units)

IV.1.2.4. DISCUSSION

IV.1.2.4.1. *Role of electron donor: acetate alone or a mixture of acetate and butyrate*

When added alone, acetate proved to be an effective electron donor for bacteria that compose the electroactive biofilm enriched from sediments in terms of lag-phase, j_{max} and CE (see Tableau IV-2). This is consistent with previous studies showing an efficient enrichment procedure from environmental samples and with acetate as the sole carbon source (Erable *et al.* 2009; Miceli *et al.* 2012; Bond *et al.* 2002; Holmes *et al.* 2004). Freguia (2010) showed that in BES fed with a mixture of more than 7 different volatile fatty acids (VFAs), acetic and propionic acids were the preferential electron donors for ARB enriched in microbial biofilms. However, when BESs were fed with a single VFA, each of these could be removed, but particularly i-butyrate did not provide significant current generation. On the other side, Liu (2005b) showed that an enriched electroactive biofilm in a MFC fed with acetate generated 66% higher power density than with butyrate suggesting that acetate is a preferential substrate. When comparing the results presented here with those of Freguia (2010) and Liu (2005b) in which acetate has been the preferential substrate for current production in BESs fed with several substrates, one could hypothesize the following: the competition with other non ARB for an specific substrate or a better degradability of acetate in comparison to other VFAs could have been hindering the ability of ARB to efficiently convert the provided substrate into current.

IV.1.2.4.2. *Role of pH: neutral and acidic conditions*

The pH value is an important parameter for microbial electron transfer. During the substrate oxidation process that occurs within the anodic microbial biofilm, electrons and protons are produced (Rabaey et Verstraete 2005). In a two chamber BES where anode/anolyte and cathode/catholyte are physically separated, e.g, by an ion exchange membrane (Rozendal *et al.* 2007), protons are released to the media and migrate to the cathode chamber to react with molecules of water and electrons resulting from the external driven flow from the anode to the cathode, finally producing reduced compounds. Therefore an unbalance of the desired pH in both anode and cathode environments could cause irreversible anodic biofilm degradation or severely affect the oxygen reduction reaction (Patil *et al.* 2011). In the work presented here, overall performance was significantly higher at pH 7.0 than at pH 5.5 when

operated under the same conditions in terms of CE (see Tableau IV-2). For reactors fed with acetate, CEs were 70% and 12% at pH 7.0 and 5.5, respectively. For reactors fed with a mix of acetate and butyrate, CEs were 11% and 21% at respective pH 7.0 and pH 5.5. These results were expected as pH in the inocula was close to 7. Hence, the microbial communities derived from the sediments have successfully adapted to this pH environment. This is also consistent with previous results in which electroactive biofilms showed a better performance in terms of CE at neutral pH. For example, Patil *et al.* (2011) showed that a higher performance was obtained at neutral pH using wastewaters as inoculum. Taking together, the results presented here corroborate the findings of a great deal of the previous BES studies comparing the effect on biofilm formation and performance. However, the saline environment proposed here for the enrichment of high current producing ARB significantly expands the possible applications of BESs.

IV.1.2.4.3. *Anodic bacterial community composition and distribution*

16S rRNA gene-based CE-SSCP fingerprints of the anodic enriched biofilms are presented in Figure IV-2. On CE-SSCP profiles, each peak is representative of a phylotype. It should be noted that the phenomenon of co-migration can generate a same peak for different, but genetically closed species. This technique provides a good overview on the microbial community structure in the biofilm. In the study presented here, the comparison between CE-SSCP profiles of the inoculum used and the one of the derived biofilms allows to clearly see the impact of the bioelectrochemical enrichment process on the anode on the microbial community (*i.e.*, anodic fixed potential, available source of electron donor, controlled temperature, etc.). Overall, CE-SSCP profiles of derived biofilms present only a few peaks which can be understood as the result of a simplified microbial community with the emergence of only one or two main dominant ARB. This illustrates the high selectivity of the procedure utilized by using a polarized electrode. A result that is consistent with previous studies showing a high bacteria selection and a decrease of diversity (Harnisch *et al.* 2011; Miceli *et al.* 2012; Torres *et al.* 2009).

As described before, the microorganisms colonizing the anodes were affiliated to delta subgroup of proteobacteria. Furthermore, from 83.5% to 98.6% of the sequences were only affiliated to two genera: *Desulfuromonas* spp. and *Geoalkalibacter* spp. Among delta subgroup of *Proteobacteria*, many species, including *Desulfuromonas* and *Geoalkalibacter*,

are able to oxidize organic compounds and reduce insoluble Fe(III) oxides at the same time (Bond *et al.* 2002; Pfennig et Biebl 1976). This study shows that the ARB found here are able to use the electrode material as an electron acceptor as they would behave in natural environment using other insoluble final electron acceptors such as iron or magnesium oxides (Greene *et al.* 2009). Consequently, it can be then proposed that in the ARB found here, especially for *Geoalkalibacter*, the capacity to reduce insoluble electron donors (such as iron oxides) does confer the ability to transfer electrons to electrode materials. Ability not always present in iron reducing bacteria (Richter *et al.* 2007).

It was previously shown that microbial composition of electroactive biofilms is highly dependent on the substrate nature (Chae *et al.* 2009; Freguia *et al.* 2010; Kiely *et al.* 2011). Freguia *et al.* (2010) showed that in an electroactive biofilm from a MFC fed with butyric acid, the microbial communities greatly differed from the MFC fed with acetic acid. A lack of *Geobacteraceae* in butyrate fed-system suggested that this group of microorganisms was not able to harvest electrons from butyrate oxidation. In contrast, a high abundance of *Pseudomonas* and gram-positive *Bacillus* species suggested that these species were probably responsible for butyric acid conversion into current (Freguia *et al.* 2010). According to Chae (2009), a higher CE was found when the anodic biofilm was enriched from an anaerobic digester sludge fed with acetate (72.3%) than with butyrate (43.0%) at pH 7. This is consistent with results at neutral pH fed with acetate (67.9±16.2 %) and with an acetate and butyrate mix (11%) (see Tableau IV-2). In Freguia *et al.* (2010), microbial communities were also different with the predominance of α, β and δ–*Proteobacteria* and *Firmicutes*, with a majority of β-*Proteobacteria* in butyrate-fed biofilm and the predominance of α, β and δ *Proteobacteria* with a majority of β and δ-*Proteobacteria* in acetate-fed biofilm. In the present study, the main dominant bacteria in each biofilm were related to *Geoalkalibacter subterraneus* and *Desulfuromonas spp.* (Tableau IV-2).

Regarding the type of ARB bacteria selected and their predominance in the anodic microbial community, the following was observed. The selection of *Geoalkalibacter subterraneus* could be due to the saline nature of the inoculum since this bacterium preferentially grows under these saline conditions (Carmona-Martínez 2013; Badalamenti 2013).

A plausible explanation for the high microbial selection in anodic biofilms found here could come from the analysis of the constant experimental conditions imposed by, e.g. a

fixed anodic applied potential, temperature and pH (among others) that assure well controlled microbial and environmental conditions for ARB to easily attach to the electrode surface while using it as a final electron acceptor (Rabaey *et al.* 2010).

Whereas *Desulfuromonas* spp has been widely described in the literature as an ARB (Bergel *et al.* 2012; Bond and Lovley 2003; Bond *et al.* 2002; Call *et al.* 2009; Dumas *et al.* 2008; Holmes *et al.* 2004; Logan and Regan 2006; Marsili *et al.* 2010; Miceli *et al.* 2012; Nevin *et al.* 2008, 2009; Qu *et al.* 2012; Wei *et al.* 2010), members of *Geoalkalibacter* genus have only recently been found in an electroactive biofilms (Miceli *et al.* 2012). Miceli (2012) showed that *Geoalkalibacter* dominated highly enriched biofilms producing significant j_{max} (4.2 to 8.9 A m^{-2}). More precisely, *Geoalkalibacter subterraneus* was found for the first time in electroactive biofilms from mixed culture. In addition, anodic electron transfer was independently evidenced by two different research groups using a pure culture of *Geoalkalibacter subterraneus* (Carmona-Martínez 2013; Badalamenti 2013).

Moreover, the random character of the dominance or co-dominance of those two bacteria (*Geoalkalibacter subterraneus* and *Desulfuromonas* spp) suggests that syntrophic interactions could exist among these two bacteria in the early stage of biofilm formation, i.e. when biofilm is sampled after the first chronoamperometric cycle as in the experimental procedure presented here.

Desulfuromonas acetoxidans an ARB thas was firstly isolated from marine sediments (Bond *et al.* 2002) is able to survive in freshwater environment (Vandieken *et al.* 2006) and is known to grow anaerobically by oxidizing acetate with the reduction of elemental sulfur or Fe(III) (Pfennig et Biebl 1976; Roden et Lovley 1993). An organism close to *Desulfuromonas acetoxidans* was repeatedly identified in enriched anodes by Bond *et al.* (2002). In their work, a pure culture of *Desulfuromonas acetoxidans* provided a current density of 0.157 A m^{-2} on a graphite electrode poised at 0.2 V/AgAgCl (Bond *et al.* 2002). Our results are consistent with that previous publication of Bond et al. (2002). However, the biofilms enriched here presented much higher current densities. This difference can be due to discrepancy between their and our experimental procedure. In the present study we work in a potential controlled device instead of an MFC.

Geoalkalibacter subterraneus is a δ-proteobacteria. It is closely related to *Geobacter* and belongs to the same *Geobacteraceae* family (90% of similarity). It is considered to be an alkalitolerant (or either alkaliphilic) and able to use a wide panel of organic acids as electron

donors such as acetate and ethanol (Greene *et al.* 2009). Only a recent study reported the finding in mixed culture of organisms related to *Geoalkalibacter subterraneus* as a dominant ARB in the population of electroactive microbial biofilms enriched from shoreline and mangrove sediments (Miceli *et al.* 2012).

The two biofilms reported by Miceli (2012) mainly containing *Geoalkalibacter* reached from 3.87 to 8.73 A m^{-2} from the first CA growth cycle on a graphite electrode polarized at -0.3 V vs. Ag/AgCl (Miceli *et al.* 2012). Our results are in good agreement with those reported by Miceli *et al*. (2012) even if applied potentials differ.. The very similar j_{max} value reached in the present study in comparison with those of Miceli *et al*. (2012) can likely be due to the saline conditions used in both cases (from 20 to 30 g_{NaCl} L^{-1}). Additionally, inocula in both studies were collected from places with a high salt content. While in Miceli (2012) salt water sediments and anaerobic sediments from a beach were used, here electrochemically derived biofilms enriched with *Geoalkalibacter subterraneus* as the main ARB were obtained from two different types of inocula: sampled sediments from a lagoon collecting wastewaters in a salt factory (Inoculum 1) and sediments from a salt lake beach (Inoculum 2).

In addition, recent electrochemical characterizations of *Geoalkalibacter subterraneus* in pure culture support our results to explain that the experimental conditions used here (such as a saline growth medium: 30 g_{NaCl} l^{-1}) can stimulate the enrichment of an halophilic ARB as *Geoalkalibacter subterraneus* (Carmona-Martínez 2013; Badalamenti 2013). Besides the study of the development of *Geoalkalibacter subterraneus* anodic biofilms under saline conditions, both research groups showed that it is indeed possible to obtain high conversion of organic matter into current even under these conditions (higher than 4 A m^{-2}).

In conclusion, the use of CE-SSCP for the analysis of the anodic microbial community provided a good characterization of the high ARB selection during electrochemically driven enrichment. Moreover, efficient electroactive biofilms were obtained in terms of significant high current densities up to 8.5 A m^{-2} and hence, high substrate conversion as coulombic efficiency up to 82.8%, clearly indicating that most of the substrate was effectively converted into current. Interestingly, among all conditions tested, species phylogenetically closed to *Desulfuromonas* spp and *Geoalkalibacter subterraneus* were the main dominant anode-respiring bacteria in all anodic electroactive biofilms, alone or together. This implies a possible symbiotic effect in the early stage of biofilm formation, an effect currently under investigation in our research group.

IV.1.3. DISCUSSION

Le but de cette étude était de sélectionner et d'identifier des bactéries électroactives en cultures mixtes dans des conditions permettant le couplage de la fermentation et de l'électrolyse microbienne. L'utilisation de la CE-SSCP pour l'analyse de la communauté microbienne à l'anode a permis de fournir une caractérisation de la structure de communauté microbienne et a montré la sélection spécifique de 1 ou 2 bactérie(s) majoritaire(s) suite à l'enrichissement sur l'électrode en condition salines et à partir de sédiments salins. Des biofilms électroactifs présentant des densités de courant élevées (jusqu'à 8,5 A/m²) ont été obtenus. Le rendement faradique a atteint jusqu'à 82,8%, ce qui indique une très bonne conversion du substrat en flux d'électrons. Ceci s'accorde avec les résultats d'Erable et al. (2009) qui ont obtenu de bonnes densités de courant (2.5 A/m²) à 20 g_{NaCl}/L en utilisant des sédiments salins comme inoculum. De la même façon, Rousseau et al. (2013) ont obtenu une augmentation des densités de courant avec l'augmentation de la salinité du milieu.

Parmi toutes les conditions testées, des espèces phylogénétiquement proches de *Desulfuromonas* spp et *Geoalkalibacter subterraneus* étaient majoritaires ou co-majoritaires dans tous les biofilms électroactifs anodiques. La forte sélection de ces espèces peut être due aux conditions expérimentales très contrôlées dont le potentiel imposé à l'anode grâce au potentiostat et le contrôle d'autres paramètres tels que le pH, la température et la composition du milieu. Ceci permet un maintien des conditions environnementales qui favorise l'attachement du biofilm en surface de l'anode et l'usage de cette électrode comme accepteur d'électrons (Rabaey et al., 2010). La présence de *Geoalkalibacter subterraneus*, qui n'a été que récemment observée dans un biofilm électroactif (Miceli et al., 2012) peut être due à la nature saline de l'inoculum, en comparaison aux études précédentes principalement inoculées avec des eaux usées et ne présentant pas une salinité élevée (Lefebvre et al., 2010). Ainsi, Miceli et al. (2012) ont montré que *Geoalkalibacter* spp prédominaient dans des biofilms présentant de fortes densités de courant (de 4.2 à 8.9 A/m²). Ces performances peuvent être attribuées au mécanisme de transfert direct d'électrons vers l'anode qui a été mis en évidence récemment par Carmona-Martínez et al. 2013 et Badalamenti et al. 2013.

Du point de vue du couplage des deux procédés de fermentation et d'électrolyse microbienne, les travaux de cette publication ont permis de d'étudier 2 conditions particulières inhérentes à chaque procédé : pH neutre ou acide et alimentation avec de l'acétate ou un mélange d'acétate et de butyrate qui correspondent respectivement aux conditions standards utilisées dans les systèmes bioélectrochimiques (Logan, 2012) et après une étape de fermentation (Guo et al., 2010).

Lorsque l'acétate a été ajouté seul comme donneur d'électrons, le rendement faradique et la densité de courant obtenus ont été plus élevés qu'avec un mélange d'acétate et de butyrate. De même, la phase de latence observée a été plus courte. Ceci vient corroborer les résultats trouvés dans la littérature avec d'autres types d'inocula et dans des conditions non salines (Liu, Cheng, et al., 2005a; Freguia et al., 2010) où l'acétate est le substrat privilégié pour la production de courant dans les systèmes bioélectrochimiques. Ceci peut être dû à une meilleure métabolisation de l'acétate en comparaison à d'autres acides organiques qui nécessitent plus d'énergie pour l'oxydation de la molécule à plus longue chaine (Venkata Mohan and Prathima Devi, 2012).

Dans nos travaux, les performances électroactives étaient significativement plus élevées à pH 7 qu'à pH 5,5 en termes de rendement faradique. Alimenté avec de l'acétate, les rendements faradiques étaient de l'ordre de 70% et 12% à, respectivement, pH 7 et pH5.5. Alimenté avec un mélange d'acétate et de butyrate, ils étaient de 11% et 21% à respectivement pH 7 et pH5,5. Ces résultats sont cohérents avec le pH d'origine de l'inoculum, plus proche de la neutralité, et donc la sélection de bactéries plus adaptées à pH 7. Ce constat s'accorde aussi avec des études antérieures où un pH neutre permet d'obtenir de meilleurs rendements faradiques (Patil et al., 2011). Enfin, une meilleure activité électroactive à pH 7 qu'à pH5,5 de *Geoalkalibacter subterraneus* et *Desulfuromonas* spp., retrouvées dans tous les biofilms, peut vraisemblablement être dûe à une adéquation avec les optima de pH de ces microorganismes. En effet, l'optimum de pH de *Geoalkalibacter subterraneus* est de 7 (Greene et al., 2009) et celui de *Desulfuromonas acetoxidans* est compris entre 7,2 et 7,5 (Pfennig and Biebl, 1976).

En conclusion, cette étude aura permis de sélectionner des bactéries électroactives adaptées à un milieu salin, l'originalité étant de les sélectionner à partir de milieux salins et anaérobies. Ainsi, une bactérie récemment identifiée comme majoritaire dans des biofilms électroactifs et caractérisée en culture pure dans des systèmes bioélectrochimiques,

Geoalkalibacter subterraneus, aura permis d'atteindre dans ces conditions spécifiques, un bon transfert d'électrons à l'anode à partir d'acétate à pH 7. De plus la forte sélection d'une ou deux espèces majoritaires et leur caractérisation comme espèces électroactives en cultures pures permet de supposer qu'elles jouent un rôle central dans le transfert d'électrons au sein du biofilm.

Suite à cette étude et étant donné la forte sélection microbienne et la structure de communauté simplifiée du biofilm, une stratégie pour pré-sélectionner les espèces électroactives à partir d'une méthode d'enrichissement sur oxydes de Fer (III) a été mise en œuvre (IV.2). Cette méthode s'appuie une autre stratégie d'enrichissement des ARB qui a été proposée mais pour laquelle la sélection microbienne n'a pas été décrite (Kim et al., 2004; Wang et al., 2010). L'enrichissement sur Fe(III) permettrait de pré-sélectionner des ARB en milieu liquide à partir d'un inoculum pour accélérer le démarrage de la MEC.

NB : Dans ce chapitre, il s'est avéré que le pH neutre était fortement favorable dans l'électrolyseur microbien. Ainsi, dans la suite de ce travail nous nous intéresserons plus particulièrement aux pH proches de la neutralité (pH 7 et pH 8) même lors du couplage de la fermentation et de l'électrolyse microbienne, et notamment dans l'étude de l'apport d'espèces exogènes issues de la fermentation sur le biofilm électroactif (Chapitre V)

IV.2. ENRICHISSEMENT DE MICRO-ORGANISMES REDUISANT LE FE(III) ET IMPACT SUR LE BIOFILM ELECTROACTIF

IV.2.1. AVANT-PROPOS

Dans la première partie de ce chapitre (IV.1), des espèces électroactives (ARB) ont été enrichies par sélection directe sur des anodes, seuls accepteurs d'électrons du milieu. Il existe cependant d'autres méthodes d'enrichissement utilisées en amont de l'inoculation et permettant d'inoculer les électrodes avec un consortium électroactif. Dans la littérature, les méthodes couramment utilisées pour pré-sélectionner des micro-organismes électroactifs avant d'inoculer une anode sont (i) soit une récupération d'effluents issus d'un BES fonctionnant en continu (Huang and Logan, 2008; Sleutels et al., 2011), (ii) soit une récupération/dispersion d'un biofilm électroactif au préalable formé dans un BES (Kim, Min, et al., 2005; Miceli et al., 2012; Ketep et al., 2013), (iii) soit une mise en présence de l'électrode à coloniser avec une électrode contenant déjà un biofilm électroactif dans son voisinage (Kim, Min, et al., 2005), soit enfin (iv) un pré-enrichissement d'un inoculum brut dans un milieu contenant des oxydes de fer (Kim, Min, et al., 2005; Wang et al., 2010). Parmi ces techniques , la plus utilisée consiste à remettre en suspension un biofilm électroactif dans un milieu liquide avant de réinoculer une électrode (Wang et al., 2010; Miceli et al., 2012). Cette technique implique la conduite préalable d'un réacteur BES pour obtenir un biofilm électroactif efficace. Par ailleurs, le procédé consistant à réaliser un enrichissement sur oxydes de fer permet de sélectionner des micro-organismes qui peuvent utiliser des oxydes de fer solides comme accepteur final d'électrons, ce qui constitue de bons candidats pour le transfert d'électrons sur l'anode dans des systèmes BES.

Le choix d'un tel procédé d'enrichissement sur oxydes de fer paraît particulièrement adapté aux microorganismes élecroactifs car l'électroactivité des bactéries implique une capacité à échanger des électrons avec un accepteur insoluble et externe comme une électrode. En effet, la première bactérie étudiée en culture pure dans ces systèmes, *Shewanella putreficiens*, avait été choisie en raison de sa capacité à réduire les oxydes ferriques solides (Kim et al., 1999). Des études ont montré que certaines bactéries isolées à partir de biofilms électroactifs étaient également capables de coupler l'oxydation de différents substrats avec la réduction des oxydes métalliques, et principalement des oxydes ferriques. Ces organismes sont appelés DMRB pour « dissimilatory metal reducing bacteria ».

Cette propriété est de plus une caractéristique physiologique des micro-organismes de la famille des *Geobacteraceae* (Holmes et al., 2002).

L'impact de cette méthode de pré-enrichissment de DMRB sur l'activité electroactive du biofilm sur anode a déjà été évalué dans des études antérieures (Kim, Min, et al., 2005; Wang et al., 2010). L'étude de Wang et al. (2010) est basée sur une sélection de DMRB en utilisant une méthode précédemment développée (Lovley and Phillips, 1986). Cette étude présente une méthode combinée de pré-sélection d'organismes électroactifs avec un enrichissement sur fer (III) à partir d'un biofilm formé au préalable au sein d'un BES. Il a néanmoins été observé une diminution de la phase de latence et une augmentation du rendement faradique de 23% à 34 % entre le biofilm d'origine (inoculé avec des boues activées) et le biofilm inoculé avec ce même biofilm enrichi. Cette étude a donc fourni de bons résultats pour améliorer les performances des BES, mais aucune information sur les micro-organismes impliqués n'a été apportée. Kim et al. (2005) ont quant à eux étudié, les effets de ces différentes stratégies d'enrichissement sur des boues anaérobies dans le cadre de la formation d'un biofilm électroactif en MFC. Après 25 cycles d'enrichissement sur fer (III), la densité de puissance fournie par le biofilm obtenu était 50% plus faible qu'avec le biofilm obtenu à partir des boues non-pré-enrichies sur Fe(III). Aucune étude sur les sélections microbiennes au cours de ces techniques d'enrichissement ont été menées. Il serait néanmoins interessant de mieux comprendre l'effet de ces techniques sur la sélection microbienne afin d'expliquer son impact sur l'activité electroactive du biofilm obtenu.

La Figure IV-3 présente le principe du procédé de pré-sélection de bactéries électroactives par enrichissement sur oxydes de Fe(III) et de son transfert avec respiration sur anode.

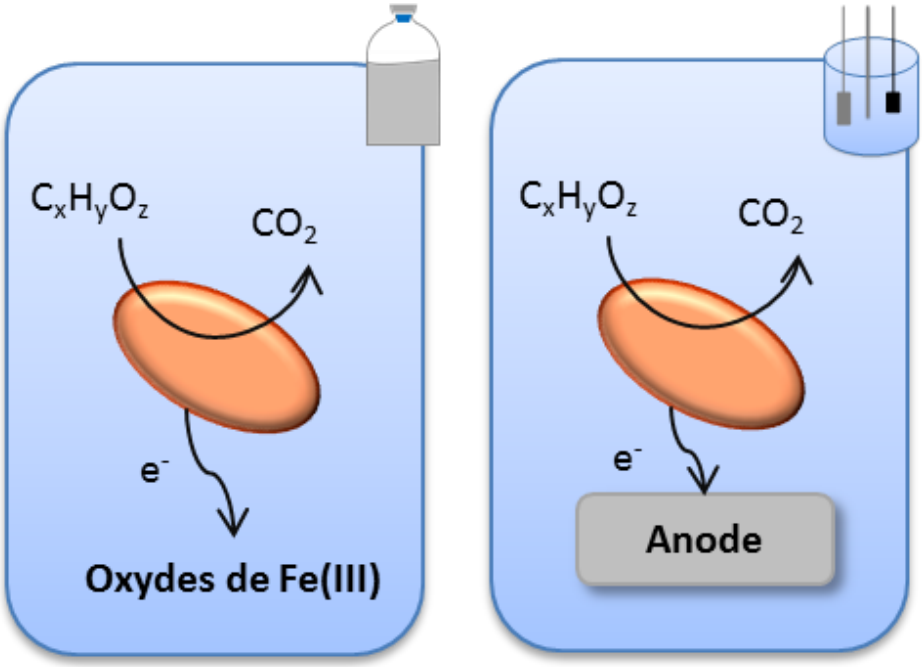

Figure IV-3 : Principe du procédé de pré-sélection de bactéries électroactives par enrichissement de DMRB sur oxydes de Fe(III).

Ce procédé tend à reproduire la réduction des oxydes métalliques, et notamment des oxydes de fer réalisée dans le milieu naturel par les DMRB (dissimilatory metal reducing bacteria) pour transférer cette capacité à transférer des électrons à un accepteur insoluble et externe à une électrode (anode).

Le but de cette étude était donc de déterminer l'effet des étapes de culture d'enrichissements successifs sur les structures de communautés microbiennes et sur les performances électroactives du biofilm. La nouveauté de ce travail est la caractérisation des communautés microbiennes au cours du processus d'enrichissement et l'utilisation de chaque étape de l'enrichissement pour produire un biofilm électroactif. Les enrichissements de bactéries électroactives sur anodes et de DMRB en cultures liquides enrichies en Fe(III) ont été réalisés dans des solutions salines et en utilisant comme inoculum des sédiments contenant des populations microbiennes déjà adaptées aux conditions de concentrations élevées de sel.

IV.2.2. ENRICHISSEMENT DE BACTERIES REDUISANT LE FE(III) POUR LA SELECTION DE MICROORGANISMES ELECTROACTIFS : CARACTERISATION DE LA SELECTION MICROBIENNE.

Iron-enrichment strategy for selecting electroactive microorganisms: New insight in microbial community characterization.

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D'après l'article en préparation pour «Bioressource Technology»

Highlights:

- Efficient iron-enrichment strategy for saline ARB pre-selection with only one step required.
- Helpful method to select *Geobacteraceae*, known as DMRB and sometimes electricigens.
- Selection of *Geoalkalibacter subterraneus*, a recently found electroactive bacteria.
- Increase and further decrease of electroactivity efficiency due to a divergence in microbial selection between DMRB and ARB.

Abstract:

Bioelectrochemical systems (BES) use the particular characteristic of electroactive biofilms to transfer electrons to a solid external acceptor (anode). Previous studies have faced some difficulties in obtaining repeatable performances from diverse natural sources of inocula, due to different compositions of microbial communities found in the mature electroactive biofilms capable of producing significant high current densities higher than 1 A/m^2, specially under high saline conditions. Different enrichment methods have been proposed for the development of efficient electroactive biofilms. Some of them are based on the hypothesis that anode-respiring bacteria (ARB) are mostly iron-respiring bacteria. Few studies have been performed focusing on the evolution of microbial communities along this enrichment strategy. In this work, we compare such an enrichment strategy in saline condition, favoring charge transport in bioelectrochemical systems, to the direct selection of

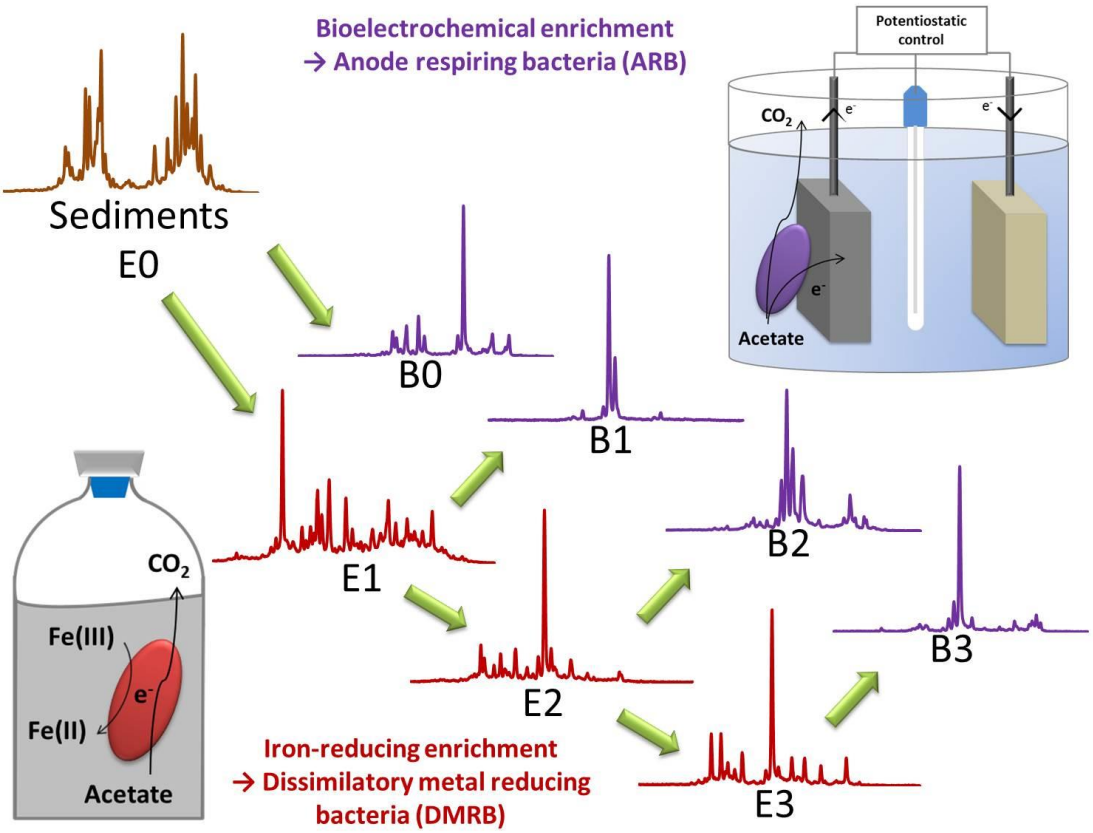
ARN on an electrode. In a first experimental stage, sediments were used to inoculate a saline medium containing Fe(III) ions. Secondly, part of the enriched community was transferred to a potentiotatically controlled system as inoculum to form an electroactive biofilm on a graphite anode. Successive enrichment steps with Fe(III) were conducted to observe the effect of the enrichment strategy on the electrochemical performance and bacterial community structure.

As result, this procedure selected *Geoalkalibacter subterraneus* after only one enrichment step, providing a practical preliminary selection tool for electroactive bacteria whereas further iron enrichment steps showed divergent selection of communities for respiration on anode or iron linked with decrease of electrochemical performances of biofilms.

Key words:

Enrichment cultures, Iron, BioElectrochemical Systems, *Geoalkalibacter subterraneus, Marinobacterium* sp.

Graphical abstract:



IV.2.2.1. INTRODUCTION

Bioelectrochemical systems (BES), such as Microbial Electrolysis Cells (MEC) or Microbial Fuel Cells (MFC), use the ability of electroactive bacteria to transfer/accept electrons to/from a solid external electron acceptor/donor such as an anode or a cathode, respectively (Pant et al., 2012). The transferred electrical current can be used for the direct production of electricity in MFCs or for the recovering of hydrogen or other products in MECs. Various anode respiring bacteria (ARB) embedded in electroactive biofilms can directly transfer electrons to/from electrode materials (Patil et al., 2012).

While microbial characterisation of several microbial BESs showed the predominance of *Geobacter sulfurreducens* in electroactive biofilms, (Holmes, et al., 2004; Badalamenti et al., 2013), analyses of diverse enriched anodic biofilms revealed many differents dominant members within these biofilm communities. An example of such microbial diversity is shown by the found ARBs usually associated to α, β, γ, δ-*Proteobacteria*, *Firmicutes*, *Acidobacteria* and *Actinobacteria* (Liu et al., 2010).

Previous studies showed the presence of anode respiring bacteria although different environmental samples were used as source of microorganisms such as freshwater and marine sediments, salt marshes, anaerobic sludge, wastewater treatment plant sludge and mangrove swamp sediments (Liu et al., 2010; Miceli et al., 2012).

In a natural environment, ARB use a variety of extracellular electron acceptors like iron and manganese (Lovley, 1993; Nealson and Saffarini, 1994). Indeed, studies showed that bacteria isolated from electroactive biofilms are able to couple the oxydation of different substrates with the reduction of metallic oxides, mainly ferric oxides. As electroactivity of bacteria involves their ability to exchange electrons with a solid electrode, the first bacteria studied in pure culture in those systems, *Shewanella putreficiens*, had been chosen because of its ability to reduce solid ferric oxides (Kim, Hyun, et al., 1999; Kim, Kim, et al., 1999). Organisms that have the ability to couple the oxydation of different substrates with the reduction of metallic oxides are called Dissimilatory Metal-Reducing Bacteria (DMRB). This property is a physiological characteristic of microorganisms from the *Geobacteraceae* family (Holmes et al., 2002).

Different enrichment techniques have been used to form high current density electroactive biofilm : the use of a continuous effluents from BES (Huang and Logan, 2008; Sleutels et al., 2011), the scraping and dispersion of electroactive biofilm (Kim, Min, et al., 2005; Miceli et al., 2012; Ketep et al., 2013), the chemical enrichment of DMRB (Kim et al., 2005; Wang et al., 2010) and the placement of the fresh electrode in the vicinity of an already active biofilm anode (Kim et al., 2005).

Previous studies have been performed to select an electroactive biofilm consortium in order to get a stable and efficient anodophilic consortium (Kim et al., 2005; Wang et al., 2010). Kim et al. (2005) studied the effect of the different enrichment strategies described above to improve electricity production in microbial fuel cells (MFC). Using anaerobic sludge as inoculum, this study shows a 50% decrease of current production with cell suspension obtained after the 25th serial transfer of the anaerobic culture in a ferric citrate medium, than when using unacclimated anaerobic sludge. As expected, the startup of the enrichment process was most successful with the biofilm grown from an inoculum originated from bioanodes (scaping/resuspending or using a nearby anodic biofilm). Wang et al. (2010) study was based on a selection of DMRB using a previously developped method for organic matter mineralization with reduction of ferric iron (Lovley et Phillips, 1986). They showed a decrease of lag phase and an increase of Coulombic efficiency from 19% and 23% with biofilm obtained respectively from activated sludge and the original biofilm to 34% with the enriched inoculum. So this study provided good results to improve BES performances but no information about the microorganisms involved.

Till now, most of the enrichment strategies consist in the re-use of an existing electroactive biofilm (Kim, Min, et al., 2005; Wang et al., 2010; Miceli et al., 2012) or effluent originated from a running BES (Huang and Logan, 2008; Sleutels et al., 2011). These techniques imply to run a BES reactor before getting an efficient electroactive biofilm. The iron-enrichment strategy could provide a less energy consuming and easier to prepare technic to pre-select ARBs. Therefore, the enrichment method in this study was chosen in order to select ARB with a capability to use solid iron oxides as electron acceptor before inoculating a BES.

In this work, an enrichment method was developed to select microorganisms able to reduce solid iron oxides as good candidates to be electroactive. The aim of this study was to determine the effect of successive iron-reducing enrichment culture stages on microbial communities and electroactivity performances of the biofilm. The novelty is the description of microbial communities during the enrichment process, an analysis usually ignored in several studies that mainly focused in improving the overal performance of the system without the full understanding obtained by a microbial analysis. The biofilm growth and liquid iron enrichment were performed in saline conditions (35 g_{NaCl}/L), using saline sediment as inoculum, already adapted to high salt concentrations conditions, to provide better conductivity in BES.

IV.2.2.2. MATERIALS AND METHODS

Sediments from a salt plant were used to inoculate a medium containing Fe(III) as sole electron acceptor in order to select bacteria reducing Fe(III) to Fe(II). The enriched community was then used as inoculum in electrochemical cells equipped with a three-electrode setup to form an electroactive biofilm on a graphite anode. This procedure was reiterated three times in four replicates to observe the effect of the succession of enrichment culture stages on both bioelectrochemical performances and electroactive biofilm community structure.

IV.2.2.2.1. *Inoculum*

The initial seed sediment was collected from a wastewater collection ditch in a salt plant, in the Salin de Saint Martin, Gruissan (France). The sediments were stored at lab temperature before inoculation. The pH of the sediments was 8.5. After centrifugation (20,000g, 10 min), the supernatant of the sediments had a salinity of 67.4 g_{NaCl}/L.

IV.2.2.2.2. *Iron-reducing enrichment cultures*

Sediments were diluted (10% v/v) in a final working volume of 500 mL with a solution containing MES buffer (100mM) and modified Starkey mineral solution (Rafrafi et al., 2013) containing the following: 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. Fe(III)-oxides (100mM) was added as electron acceptor and acetate (10mM) as electron donor. NaCl and deionized water were added to get a final NaCl concentration of 35 g/L to assure saline conditions. The initial pH was adjusted to 7 using NaOH (1M). To ensure anaerobic conditions, each bottle was flushed with high purity nitrogen gas for 30 minutes after inoculation. Composition of headspace was checked and oxygen relative abundance was less than 1.0%. Reactors were incubated at 37°C till the whole acetate consumption.

Two-milliliter aliquots were periodically collected and centrifuged (20,000g, 10 min). Pellets and supernatant were stored at -20°C for further chemical and microbiological analyses.

The conversion of Fe (III) to Fe (II) was assessed spectrophotometrically with a ferrozine assay of HCl-extractable Fe(II) (Lovley and Phillips, 1988, Wang et al., 2010).

IV.2.2.2.3. *Bioelectrochemical enrichment*

The sediments and each step of the iron-enriched culture were used to grow an electroactive biofilm in four replicates in a three electrode system (*Figure IV-4*). This electrochemical reactor were carried out under potentiostatic control (VSP Bio-Logic SA) monitored with a computer (EC Laboratory v.10.1 software, Bio Logic SA). The arrangement, as previously described (Ketep et al., 2013), consisted of a graphite working electrode, a saturated calomel electrode (SCE) as reference electrode and a platinum grid counter electrode. The anodes were geometrically defined graphite planar working electrodes with the following dimensions: 2.5 x 2.5 x 0.2 cm (Goodfellow). Electrical connection consisted in 2 mm diameter and 12 cm long titanium rods (Goodfellow). The working electrodes were cleaned before use using a P800 sandpaper and rinsed with 99% purity ethanol. Cathode (counter electrodes) were Platinum Iridium grid (90%/10%) (Heraeus) cleaned by heating in a blue flame. The graphite working electrode potential was fixed using a SCE electrode (+0.2V vs SCE).

Inocula were added to the culture medium (10% v/v in a final working volume of 500mL) containing 50mM MES buffer and modified Starkey mineral medium described above.

Electroactive biofilms were collected for microbial characterization for each replicate after the whole acetate consumption using a scalpel.

Current production was monitored online using a potentiostat (EC Laboratory v.10.1 software, Bio Logic SA). Maximum current densities j_{max} (/m²) of mature microbial biofilms were calculated considering the total immersed electrode surface area since electroactive biofilms covered both sides of the electrode with the same microbial community profile (CE-SSCP patterns)

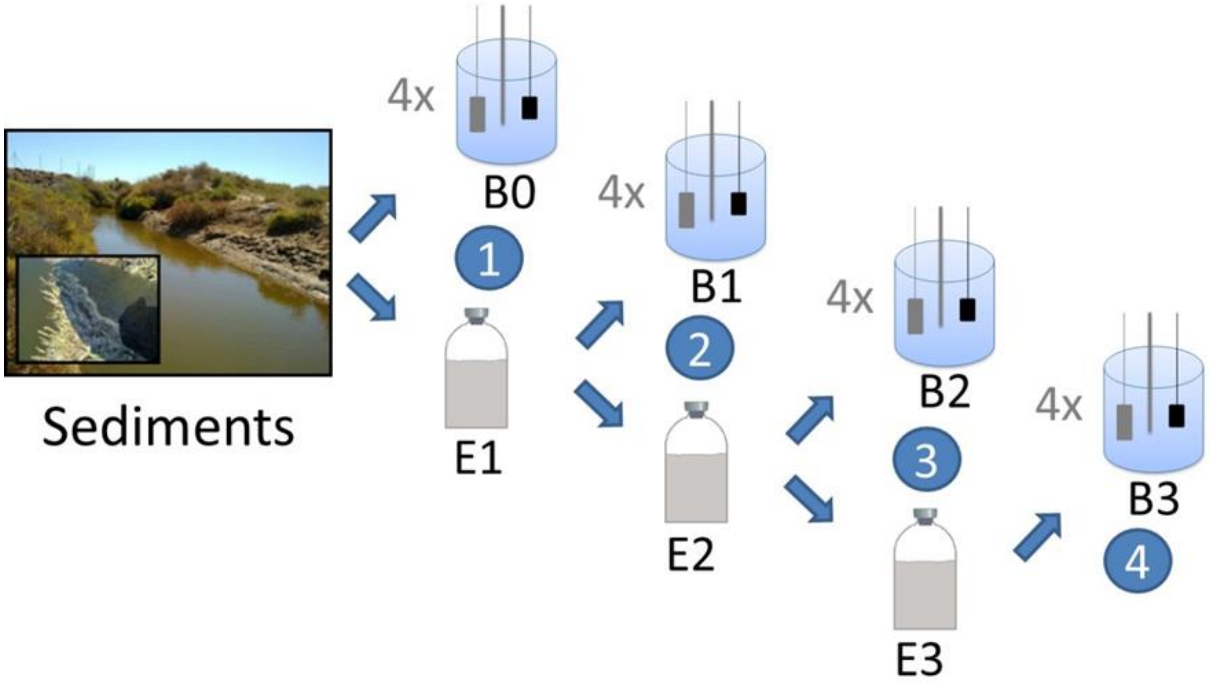


Figure IV-4 : Scheme of successive enrichments steps to grow electroactive biofilms.

Sediments were used to inoculate a liquid enrichment medium containing Fe(III) in which a bacterial community E1 was obtained and 4 replicates of electrochemical systems equipped with a 3 electrode setup, in which bacterial community B0 was obtained. This procedure was reiterated using the successive enriched communities E1, E2 and E3 to grow respectively B1, B2, B3 biofilms.

IV.2.2.2.4. *Chemical analyses*

Acetic acid (C2), was analyzed with a gas chromatograph equipped with a flame ionization detector (GC-3900 Varian). An internal standard was mixed with each sample. An autosampler was used to perform analysis. A syringe automatically takes 0.5µL of the mix and places it in the injector, heated to 250°C. The column used is an ELITE FFAP, 15000x0.53 mm (Perkin Elmer), the temperature is maintained at 120°C. The carrier gas is nitrogen (N_2) and the flame ionization detector gas is hydrogen (H_2).

IV.2.2.2.5. *Data analysis*

To characterize electroactive performance of the biofilm, transmitted charge for each reactor was fitted to a modified Gompertz equation as previously described (Quéméneur et al., 2011) (*Figure IV-5*):

$$Q(t)=Q_{max}.\exp(-\exp(V_{max}.\exp(1)/ Q_{max} (\lambda-t)+1))$$

Where Q: Charge (C) ; V_{max}: maximum transferred charge rate (C/day); λ: lag phase (day) ; t: incubation time (day),

Coulombic efficiency, i.e. the amount of coulombs trapped in electrical current generation relative to the maximum reachable assuming the whole substrate oxidation, was calculated for each reactor according to (Call et al. 2009).

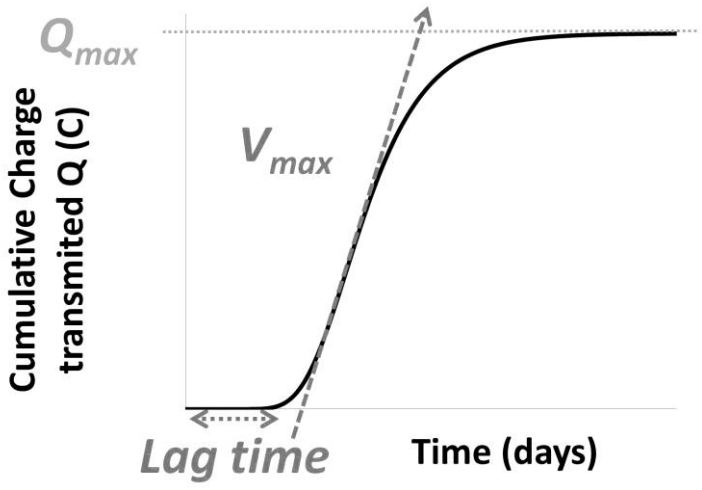


Figure IV-5 : Representation of electron transfer dynamics from a mixed culture cultivated with acetate as substrate in electrochemical systems equipped with a 3 electrode setup.

The estimated values correspond to the charge Coulombic efficiency (%), the maximum current production rate (C/d) and the lag phase time (day), respectively.

IV.2.2.2.6. *DNA extraction and PCR amplification*

Molecular analyses of bacterial communities were performed after acetate consumption in each biofilm reactor and enriched culture. Genomic DNA was extracted and purified from the biofilm using a previously described protocol (Godon et al., 1997). Then, the total extracted DNA was purified with a QiAmp DNA microkit (Qiagen, Hilden, Germany). To evaluate success of DNA extraction, DNA amount and purity were confirmed by spectrophotometry (Infinite NanoQuant M200, Tecan, Austria). Then, 16S rRNA genes were

amplified with using universal primers for bacteria W49 (5'-ACGGTCCAGACTCCTACGGG-3' *Escherichia coli* position 330) (Wéry et al., 2008) and 5'-fluorescein phosphoramidite named W104 (5'-6FAM-TTACCGCGGCTGCTGGCAC-3' *Escherichia coli* position 533) (Wéry et al., 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 carried out in Mastercycler thermal cycler (Eppendorf). The 16S rRNA genes were amplified as follows : initial denaturing 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.

IV.2.2.2.7. *CE-SSCP electrophoresis and statistical analyses*

PCR products were separated by capillary electrophoresis single-strand conformation polymorphism (CE-SSCP). With this technique, DNA fragments of the same size but with different compositions are separated (Wéry et al., 2008).

An appropriate dilution of PCR products (1µL) was mixed with 18.925 µL of formamide and 0.075 µL of internal standard GeneScan ROX (Applied Biosystems). Then, samples were heat-denatured at 95°C for 5 min and immediately re-cooled on ice for 5 min. CE-SSCP electrophoresis was carried out in a ABI Prism 3130 genetic analyzer (Applied Biosystems) with four 50 cm capillary tubes filled with a conformation analysis polymer (Applied Biosystems) into the corresponding buffer and 10% glycerol. Samples have been eluted at 12kV and 32°C for either 30 min (Wéry et al., 2008)**.**

To consider inter-sample electrophoretic variability, the CE-SSCP profiles were aligned with the internal standard (ROX). CE-SSCP profiles were then normalized using the *Statfingerprints* library (Michelland et al., 2009) from R software version 2.9.2 (R. Develpment Core Team, 2010) with a standard procedure described elsewhere (Fromin et al., 2007). To obtain similarity matrices, genetic distances between bacterial communities were evaluated using Euclidean distances and were statistically compared using a principal component analysis (PCA).

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

IV.2.2.3. RESULTS AND DISCUSSION

IV.2.2.3.1. *Iron-reducing enrichment cultures*

IV.2.2.3.1.1 *Iron conversion*

Electron transfer from acetate to the reduction of Fe(III) into Fe(II) was assessed. This ratio increased progressively from 4.7 to 8.0 $mol_{Fe(II)}/mol_{acetate}$ which corresponded to the maximum stoichiometric conversion rate, for the chemical enrichments E1 and E3, respectively. Wang et al. (2010) reported a similar trend with iron reducing enrichment cultures of successive electroactive biofilms operated with mixed cultures. They reported a conversion of 7.6 $mol_{Fe(II)}/mol_{acetate}$. Roden & Lovley (1993) previously reported the same conversion rate (7.6 $mol_{Fe(II)}/mol_{acetate}$) in an iron reducing pure culture of *Desulfuromonas acetoxidans*, also known as an electroactive bacteria (Bond et al., 2002). The latter value is very close to the theoretical value of electrons released after complete acetate oxidation. The values obtained in the present study are in strong accordance with these previous studies. Moreover, the increase in acetate conversion for Fe(III) reduction into Fe(II) ratio in this present study suggests that successive iron-reducing enrichment steps led to a microbial community more efficient for this respiration pathway for each step. Therefore, the bacteria selected along the enrichment steps used an increasing part of the electrons released from acetate breakdown for iron reduction instead of other purposes such as biomass production (Logan, 2009).

IV.2.2.3.1.2 *Microbial community*

A significant bacterial selection occurred all along the iron-reducing enrichment steps with the predominance of two bacterial species sub dominant in the original microbial community (see Figure IV-7). Their relative abundance percentage can be found in Figure IV-6A. *Geobacteraceae* species (including *Geoalkalibacter subterraneus* and a *Geobacteraceae* species) were selected with a decrease of the abundance of *Clostridiaceae* species along the enrichment steps. *Geoalkalibacter subterraneus* is known as ARB (Miceli et al., 2012; Badalamenti et al., 2013; Carmona-Martínez et al., 2013) and DMRB (Holmes et al., 2002; Greene et al., 2009), which is in agreement with the targeted effect of our iron-reducing enrichment.

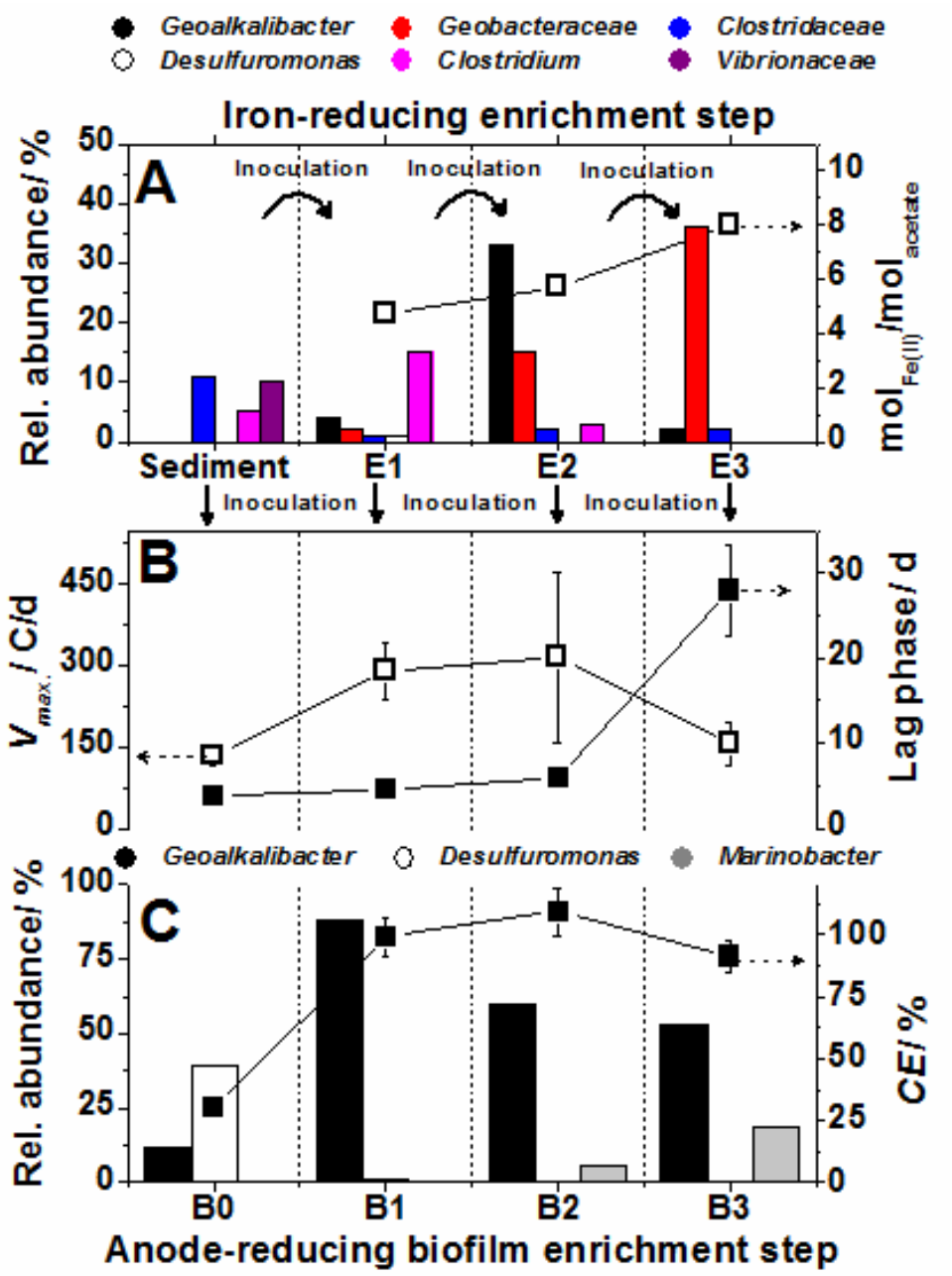


Figure IV-6 : Iron/anode enrichment parameters and relative abundance of main bacteria in microbial communities

(found as >9% in one of enriched bacterial community) identified by pyrosequencing analysis in enrichment cultures wherein E1, E2 and E3 correspond respectively to the 1st, 2nd and 3rd enrichment steps and biofilms wherein B0, B1, B2, B3 correspond to the biofilms obtained respectively with sediments, E1, E2, E3 as inoculum. Relative abundance was defined as the number of sequences affiliated with that taxon divided by the total number of sequences. (A) Relative abundances of main bacteria (found at more than 9 % in at least 1 bacterial community) in E1, E2, E3 and their abundances in the original inoculum E0. Conversion acetate electrons into Fe(II) is presented in $mol_{Fe(II)}/mol_{acetate}$ (B) maximum charge production rate (V_{max} in C/d) and lag phase (in days) before exponential phase of charge curve of anodic biofilms (C) Coulombic efficiency (CE in %) and relative abundance of main bacteria in the bioelectrochemical enriched biofilms (found at more than 9 % in at least 1 bacterial community).

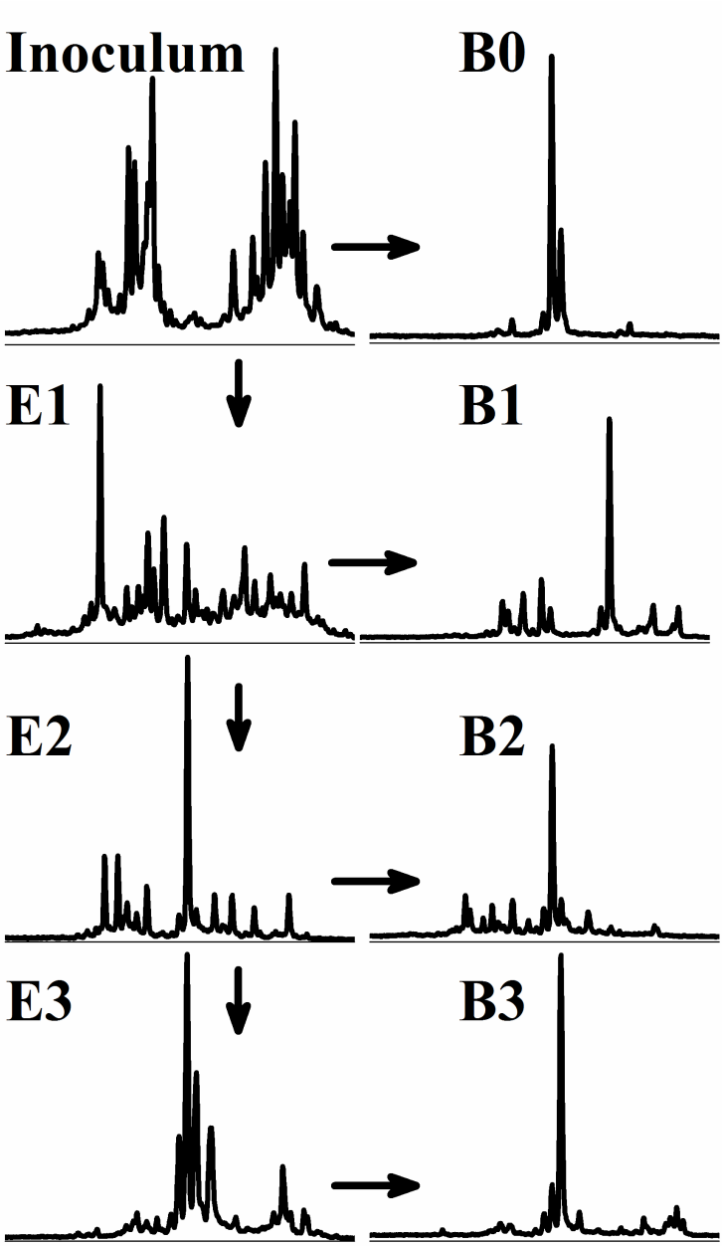


Figure IV-7 : CE-SSCP profiles based on 16S rRNA gene fragments retrieved from sediment, enriched cultures (E1, E2, E3) and biofilms (B0, B1, B2, B3) mixed cultures.

The profiles correspond to samples taken before inoculation of electrochemical cells for sediments, E1, E2 and E3 or at the maximum charge production for biofilms (B0, B1, B2, B3). The X and Y axes of each peak represent respectively the relative peak electrophoresis migration distance and the relative peak intensity (Arbitrary Units). Arrows presents the enrichment of microbial communities.

IV.2.2.3.2. *Anode-reducing enrichments*

IV.2.2.3.2.1 *Maximum electron transfer rate (V_{max})*

The maximum electron transfer rate (V_{max}), described in *Figure IV-5*, represents the maximum rate of electron transfer from the anode to the cathode per unit of time performed by the biofilm. This parameter represents the capacity of electron transfer of the biofilm. From *Figure IV-6*B it can be clearly seen that V_{max} increased with the anode-reducing biofilm enrichment steps up to 300 C/d in B2. However, for enrichment step E3 the biofilm formed B3 V_{max} significantly decreased down to 150 C/d. This is likely due to the selection of less or non-electroactive bacteria after several enrichment steps. These bacteria indeed might outcompete for the substrate consumption with electrogenic biofilm. These results might also be due to a dilution effect of the highly electroactive bacteria present in the sediment among the iron-reducing enrichment cycles procedure.

IV.2.2.3.2.2 *Lag phase*

The lag phase is presented in Figure IV-6B and corresponds to the time before reaching the exponential phase of the charge curve. In the experiments presented here, the lag phase of the anodic biofilms was slightly extended for enrichments B0, B1 and B2. This means that the anodic biofilm formation for all experimental units took about 4 to 6 days to begin. This is in good agreement with previous experiments on anodic biofilm formation (Wang et al., 2010; Patil et al., 2011). In contrast to B0, B1 and B2, the B3 experiment showed a very long lag phase of about 28 days likely due to a difference between bacteria selected on the anode biofilms and in iron-reducing enrichment. Indeed, a possible explanation for this increase may be the lack of electroactive bacteria in the further iron-reducing enrichment steps due to dilution among successive iron-enrichment steps. This dilution effect resulted in a longer lag phase for electron transfer to the anode (Wang et al., 2010).

IV.2.2.3.2.3 *Coulombic efficiency (CE)*

The fraction of electrons available in the substrate that ends up as electrical current in the system is denominated as Coulombic efficiency (CE) (Sleutels et al., 2011). CE is an indication of the actual amount of coulombs harvested from substrate oxidation and transferred by the biofilm to the anode. Consequently, increasing the Coulombic efficiency is 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, and afterwards into valuable chemicals, to create an economically feasible process (Hamelers et al., 2010). In our study, the CE increased with the iron-reducing enrichment procedure inocula (E1) from 30.4±3.8% (B0) to 99.0±7.5% (B1) and 109,1±9.9% (B2) and then rather decreased to 97.1±6.3% (B3 inoculated with B3). Interestingly, the CE increased continuously to reach the maximum value of substrate conversion as it was observed for the rate of acetate conversion for Fe(III) reduction into Fe(II). These observations are evidences of high efficiency of the enrichment procedures based on both anode and iron reduction. The Coulombic efficiency of B2 was higher that 100%. This was likely due to the oxidation of hydrogen produced at the anode (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. at the maximum of current production, biogas was composed of CO_2 8.4 %, H_2 42.12 %, N_2 33.26 % and CH_4 11.30 %. This increase in CE is consistent with Wang et al. (2010) who reported an increase of Coulombic efficiencies in a biofilm due to the iron-reducing enrichment procedure. This increase can be due to the selection of electroactive bacteria and to the decrease in the relative abundance of non ARB initially present in the inoculum of the anodic biofilm. Wang et al. (2010) study presented a similar trend with an increase of Coulombic efficiency from 19% and 23% with activated sludge inoculated biofilm and the original scrapped/resuspended biofilm, respectively, up to 34% with the iron-enriched biofilm. This can also be due to the absence of electron acceptors initially present in the sediments environment that were removed with dilution of the enriched inoculum. In our work, a single step of iron-enrichment seems to be efficient to achieve high Coulombic efficiency, i.e. to successfully convert acetate into electrons.

IV.2.2.3.2.4 *Current density*

Interestingly, no increase in maximal current density (j_{max}) was observed after the first anode-reducing biofilm step. Although CEs increased progressively through enrichment steps from B0 to B2, the current density decreased from B0 to B3 with respectively 4.5±0.7 A/m² and 1.6±0.8 A/m². These results suggested the presence of essential nutrients in the inoculum E0 that are required to achieve a high current density of B0. Their removal hindered the biofilm to produce as much current density as in B0. The diluting effect of E0 along the iron-enrichment steps supports such hypothesis of ARB starvation. There are similarities between this phenomenon shown with successive iron-reduced enrichment

cultures and previous studies where current densities had not been recovered among successive batch cycles of the same biofilm (Holmes, Nicoll, Bond, et al., 2004b; Badalamenti et al., 2013; Parameswaran et al., 2013). Moreover, in Kim et al. (2005) study, the voltage obtained in a MFC inoculated with a 25th time iron-enriched inoculum was 50% lower than the one obtained with the original unacclimated inoculum.

IV.2.2.3.2.5 *Microbial community*

IV.2.2.3.2.5.1 Selection in biofilm microbial community

Microbial community structures presented a high decrease of diversity in iron-reducing enrichment cultures and in anode-reducing enrichment biofilms compared to the inoculum (see Figure IV-7). The most striking result to emerge from the CE-SSCP profiles is that the anodic bacterial community was composed of only 1 or 2 dominant species. This finding is in agreement with previous studies in which systems inoculated with sediments presented a high simplification of the bacterial community in anode-reducing enrichment processes and a majority of *δ-Proteobacteria* (Bond et al., 2002; Tender et al., 2002; Miceli et al., 2012). However several studies reported a highly diverse microbial community with bacteria belonging to *Firmicutes*, *γ-*, *β-* and *α-Proteobacteria*, especially for systems inoculated with non-saline sediments (Rabaey et al., 2004; Lu et al., 2012). In conclusion, our results suggest that very well controlled (pH=7, 37°C, fixed potential) and also very specific (salinity, medium composition) conditions caused a high selection of bacteria both in anode-respiration enrichment process and in liquid iron-reducing enrichments.

IV.2.2.3.2.5.2 Divergence of bacterial communities

Figure IV-8 presents the results of a principal component analysis (PCA) where the genetic distances between CE-SSCP profiles representing the bacterial communities were compared, according to the experimental conditions. The profiles are compared by using the Euclidian distances between CE-SSCP patterns. On PCA, the closer are the points, the more similar are the microbial communities. In this PCA, both iron-reducing enrichment cultures (squares) and electrochemically enriched biofilms (circles) are presented. In addition, a trend of the positive correlation between the optimum performances (*Figure IV-6*) and the similarity between chemical and electrochemical microbial community structure was observed. (*Figure IV-8*). For example, for the best performing biofilm (B2) in terms of Coulombic efficiency, the corresponding enrichment microbial community, i.e. inoculum E2, is very close to the biofilm

community profile (*Figure IV-8*). Furthermore, the divergence in microbial community composition between a liquid enrichment culture and the biofilm produced from this culture was likely linked to the increase of the lag phase (*Figure IV-6*), especially for biofilm B3 inoculated with the enrichment culture E3.

Regarding the differences in microbial communities, a correlation was observed between microbial community structures in iron/anode reducing enrichment cultures and changes in anode-reducing performances. This suggests that there was a difference between extracellular electron transfer respiration in anode-reduction enrichment and in iron-reduction enrichment cultures. The two mechanisms probably require slightly different physiologies and with selecting the bacteria on a DMRB media, a specific consortium for Fe(III) respiration was selected that does not fit for a respiration on anode. Nevertheless, one step of enrichment is efficient to reach successful substrate conversion in terms of Coulombic efficiency as described above.

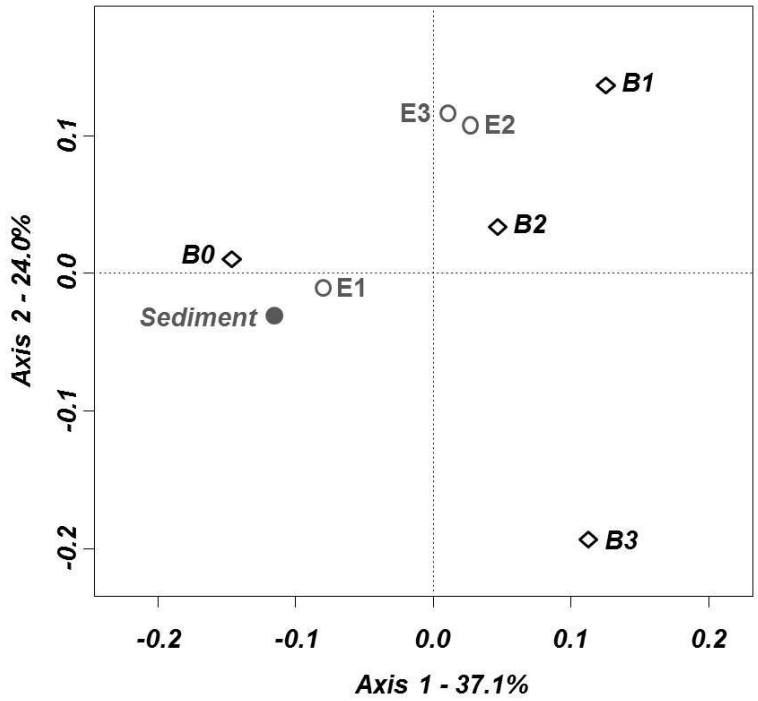


Figure IV-8 : Principal Component Analysis (PCA) biplot of CE-SSCP profiles.

For each biofilm (B0, B1, B2, B3), an average of the 4 replicate profiles is presented (◊). CE-SSCP profiles of the sediment originate inoculum E0 (●) and of iron-enrichments microbial communities (E1, E2 E3) are also presented (○). The first two principal components (Axis1 and Axis2) explained 61.1% of the genetic variation.

IV.2.2.3.2.5.3 Identification of *Desulfuromonas* sp. and *Geoalkalibacter subterraneus*

In electroactive biofilms (*Figure IV-6*C), *Desulfuromonas* sp. was selected in B0. This finding is consistent with other research where a majority of δ-*Proteobacteri*a was found in anode-reducing biofilms inoculated with sediments (Bond et al., 2002; Tender et al., 2002; Miceli et al., 2012) with systems inoculated with sediments. Bond et al. (2002) showed that 71% of the 16S RNA gene sequences from a MFC anodic biofilm inoculated with marine sediment belonged to δ-*Proteobacteria* and 70% from a single cluster of bacteria that belongs to the family *Geobacteraceae*, related to *Desulfuromonas acetoxidans*. In similar conditions, Tender et al. (2002) found 76% of the 16S rRNA gene sequences were δ-*Proteobacteria* and 59% with more than 95% of similarity to *Desulfuromonas acetoxidans.*

Interestingly, *Geoalkalibacter subterraneus* was selected as main bacteria in biofilm B1 to biofilm B3, and the most dominant microorganisms colonizing the anodes were affiliated to *Desulfuromonas* spp. and *Geoalkalibacter* spp. genera in B0. Within the delta subgroup of *Proteobacteria*, many species, including *Desulfuromonas* and *Geoalkalibacter*, are able to oxidize organic compounds and reduce insoluble Fe(III) oxides at the same time (Pfennig and Biebl, 1976; Bond et al., 2002). This study shows that the ARB found here are most probably able to transfer the ability that they have in natural environment using other insoluble final electron acceptors such as iron or magnesium oxides to the electrode materials as final electron acceptors (Richter, Lanthier, Nevin, Lovley, et al., 2007). Interestingly, in all biofilms of our study, an enrichment of *Geoalkalibacter subterraneus* was observed. Consequently, it can be assumed that for the ARB found here, and especially for *Geoalkalibacter subterraneus* that provided good electron transfer in biofilm B1, the ability to reduce insoluble electron acceptors (such as iron oxides) does confer the capacity to transfer electrons to an anode. However this ability is not always transferrable (Richter et al., 2007).

While *Desulfuromonas* spp. has been widely described in the literature as an ARB (Bond et al., 2002; Dumas et al., 2008b; Nevin et al., 2008, 2009; Call et al., 2009; Marsili et al., 2010; Wei et al., 2010; Nercessian et al., 2012), only one recent study found bacteria genetically related to *Geoalkalibacter subterraneus* as a dominant ARB in mixed culture in the microbial population of electroactive microbial biofilms inoculated with shoreline and mangrove sediments (Miceli et al., 2012). Miceli (2012) and co-workers showed that *Geoalkalibacter* was dominant in highly electroactive biofilms exhibiting j_{max} up to 8.9 A/m².

In addition, a mechanism of direct anodic electron transfer was recently evidenced by two different research groups using a pure culture of *Geoalkalibacter subterraneus* (Carmona-Martínez 2013; Badalamenti 2013). Those studies in pure cultures provided electrochemical characterizations of *Geoalkalibacter subterraneus* (Carmona-Martínez 2013; Badalamenti 2013). In those studies, *Geoalkalibacter subterraneus* provided high current densities of 4.68 ± 0.54 A/m² and 3.3 A/m², respectively. These demonstrate that other members of the *Geobacteraceae* family than the ARB model *Geobacter sulfurreducens* have the ability to produce high current densities in pure culture. This provides new insight for research in extracellular electron transfer respiration as *Geoalkalibacter subterraneus* is an efficient new anode respiring bacteria that presents good skill to favour this mechanism (Miceli et al., 2012; Badalamenti et al., 2013; Carmona-Martínez et al., 2013). Moreover, we show that only one step enrichment culture to select DMRB based on iron respiration is efficient to select *Geoalkalibacter subterraneus*, known as both DMRB (Holmes et al., 2002; Greene et al., 2009) and ARB (Carmona-Martínez 2013; Badalamenti 2013).

IV.2.2.3.2.5.4 *Geoalkalibacter* vs. *Geobacteraceae*

In this study, selection of *Geoalkalibacter subterraneus* from iron-enrichment culture permitted to increase biofilm performances from the biofilms inoculated with the first iron-reducing enrichment (B1) compared to the biofilms inoculated with the sediment (B0) (*Figure IV-6*). The most abundant bacterium of the biofilm obtained with the first enrichment E1 was 99% similar to *Geoalkalibacter subterraneus*, and 89% to the *Geobacteraceae* detected as most abundant in the second enrichment step E2. This bacterium belonging to *Geobacteraceae* presents good skills to reduce Fe(III) as many *Geobacteraceae* but has probably a low capacity to transfer this property to graphite anode respiration, as previously described for other DMRB (Richter et al, 2007). Therefore, the emergence of this *Geobacteraceae* in the detriment of *Geoalkalibacter subterraneus* can be correlated to the decrease of electroactive efficiency of biofilms after the first enrichment step.

IV.2.2.3.2.5.5 Emergence of *Marinobacterium* sp.

An increasing divergence is observed between the bacterial communities obtained in liquid enrichment cultures and the biofilms grown from these enrichments. The emergence of *Marinobacterium* sp., a δ-*Proteobacteria*, obligate aerobe and not-known as electroactive bacteria, in biofilms B3 obtained after the third iron-enrichment step (E3) is associated to the decrease of electroactivity of biofilms after the second enrichment step.

Marinobacterium sp. was retrieved in a previous study of efficient electro-active biofilms formed from natural biofilms scraped from a floating bridge (Erable et al., 2009) and associated with bacteria related to *Bacteroidetes*, *Halomonas*, certain species of *Idiomarina* and *Pseudoidiomarina.* To the best of our knowledge, this bacterium has not been studied in pure culture in electrochemical systems or reported as a dominant population in any efficient electroactive biofilm. Marinobacterium species have been isolated from various sources related to marine environments such as marine sediment, coastal areas, and coral mucus (Choi et al., 2012). *Marinobacterium* sp. were reported to produce siderophore, Fe(III) chelators for iron capitation (Weber et al., 2006; Amin et al., 2012). This property could explain its selection in an iron-enriched medium.

IV.2.2.4. CONCLUSION

After one step of iron-enrichment in a liquid medium containing Fe(III) as the lonely electron acceptor, the selection of *Geoalkalibacter subterraneus*, a recently characterized electroactive bacterium that provides good electroactive skills and which is adapted to saline conditions was obtained. Then, the divergence in microbial community composition, associated with a decrease of electroactive efficiency, suggests that respiration on Fe(III) and on anode are different and presumably require rather dissimilar skills. The findings of this study suggest that only one iron-enrichment step should be proceeded using this strategy

IV.2.3. DISCUSSION

Une étape d'enrichissement a permis de sélectionner *Geoalkalibacter subterraneus*, espèce électroactive nouvellement caractérisée, et d'augmenter le rendement faradique du biofilm. Ceci peut être dû à la suppression ou plutôt à la dilution de microorganismes qui pourraient être en compétition pour le substrat dans le milieu et d'accepteurs d'électrons alternatifs présents dans les sédiments. Cependant l'analyse des communautés microbiennes du biofilm aux différentes étapes d'enrichissement montre qu'il existe dès le premier biofilm B1 obtenu avec l'enrichissement E1 un changement dans la composition microbienne par rapport au biofilm B0 obtenu avec les sédiments. En effet, alors que *Desulfuromonas acetoxidans* était majoritaire dans B0, c'est *Geoalkalibacter subterraneus* qui devient majoritaire dès le premier enrichissement dans B1. Ceci peut être attribué à la sélection préférentielle de *Geobacteraceae* pour la respiration sur Fe(III) dans les enrichissements, propriété reconnue chez les espèces de la famille des *Geobacteraceae*.

Alors que l'espèce la plus abondante dans le biofilm B1 obtenu à partir du premier enrichissement est à 99% similaire à *Geoalkalibacter subterraneus*, l'espèce obtenue par enrichissement à partir de la deuxième étape d'enrichissement (E2) puis majoritaire dans le troisième enrichissement (E3) appartient à la famille des *Geobacteraceae* et n'est qu'à 89% similaire à *Geoalkalibacter subterraneus*. Il est donc probable que les étapes successives d'enrichissement mènent à la sélection d'une espèce appartenant aux *Geobacteraceae*, mais qui n'est pas capable, contrairement à *Geoalkalibacter subterraneus*, de transférer cette propriété de transfert d'électrons à la respiration sur anode comme cela a été précédemment montré pour d'autres organismes (Richter et al, 2007).

La divergence de sélection microbienne au cours des étapes d'enrichissement associée avec la diminution de l'efficacité électroactive du biofilm laisse supposer que les respirations sur Fe(III) requièrent des propriétés différentes chez les microorganismes et mènent à la sélection de microorganismes plus adapté à la respiration sur Fe(III) que sur anode de graphite à potentiel imposé. Ceci est en accord avec les résultats de Kim et al. (2005) qui ont noté une diminution de 50% des performances électroactives (en termes de densité de puissance produite en MFC) après 25 étapes d'enrichissemnet sur Fe(III). Les résultats de notre étude suggèrent qu'une seule étape d'enrichissement sur Fe(III) soit réalisée dans le but d'inoculer une anode. Ceci afin de ne pas obtenir une communauté microbienne trop spécialisée dans la respiration sur Fe(III). Cette méthode d'enrichissement est une technique

simple qui ne nécessite pas la mise en œuvre d'un système bioélectrochimique au préalable. Elle peut constituer une étape de pré-sélection en milieu liquide de bactéries électroactive pour inoculer une anode.

Cette méthode a ici été utilisée sur un inoculum déjà connu et qui contenait *Geoalkalibacter subterraneus.* Il serait intéressant de vérifier s'il est possible d'enrichir de bactéries électroactives avec d'autres sources d'inoculum moins favorables.

IV.3. DYNAMIQUE DE COLONISATION DES ELECTRODES

IV.3.1. AVANT-PROPOS

Dans la littérature, lors d'expériences réalisées en batch, le biofilm anodique formé est prélevé au maximum de production de densité de courant, c'est à dire quand l'électroactivité du biofilm est maximale (Torres et al., 2009; Harnisch et al., 2011; Miceli et al., 2012), ou à la fin du batch, correspondant à la consommation totale du substrat (Parameswaran et al., 2010; Liu, Wang, et al., 2012).

De même, dans les expériences conduites dans le cadre de cette thèse, nous avons prélevé le biofilm aux deux stades de croissance décrits précédemment, en utilisant le même inoculum (sédiments des salins de Saint-Martin, Gruissan) comme indiqué en *Figure IV-9* :

1) Au maximum de densité de courant pour les biofilms B7, B8 et B9 dans la partie IV.1 (Sélection et identification de microorganismes électro-actifs)
2) A consommation totale du substrat pour le biofilm B0 (formé dans les mêmes conditions que B7, B8 et B9 décris précédemment) dans la partie IV.2 (Enrichissement de micro-organismes réduisant le Fe(III) et impact sur le biofilm électroactif)

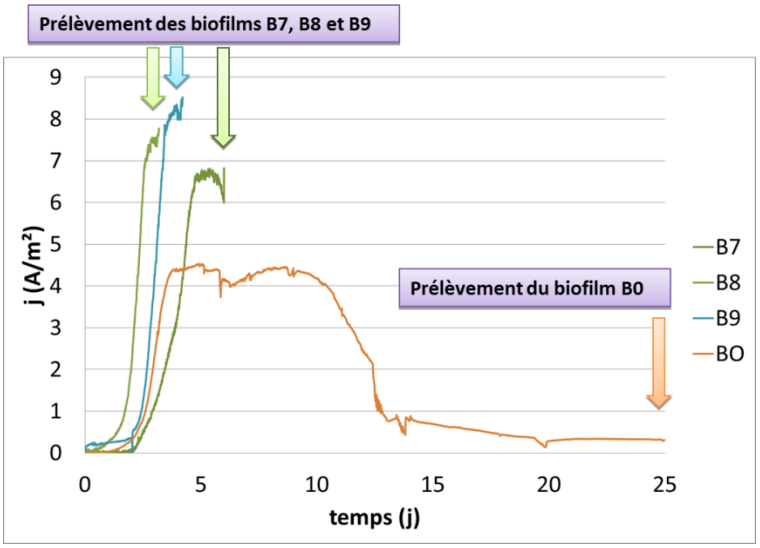


Figure IV-9 : Chronoampérométries réalisées sur les biofilms B7, B8 et B9 en partie Partie IV.1 et B0 en partie IV.2.

Les moments de prélèvements sont indiqués par des flèches. B7, B8, B9 ont été prélevés à leur maxima de production de courant et B0 a été prélevé après consommation totale du substrat.

Le *Tableau IV-3* présente les bactéries prédominantes dans les biofilms électroactifs dans chacun des cas évoqués précédemment (B0, B7, B8, B9).

Tableau IV-3 : Bactéries majoritaires, en abondances relatives, dans les biofilms B7, B8 et B9 en partie IV.1 et B0 en partie IV.2. Les densités de courant et rendement faradiques sont également indiqués en A/m² et %.

Biofilms	**j_{max}/ A m^{-2}**	**CE/ %**	**Bactéries majoritaires identifies dans le biofilm (Pourcentage d'abondance relative)**
B7 (Partie IV.1)	6.7	60	*Geoalkalibacter subterraneus* (72,86%)
B8 (Partie IV.1)	7.7	53	*Geoalkalibacter subterraneus* (20,08%) *Desulfuromonas* spp. (58,18%)
B9 (Partie IV.1)	8.5	77	*Geoalkalibacter subterraneus* (91.24%)
B0 (Partie IV.2)	4.5	30	*Geoalkalibacter subterraneus* (12,00%) *Desulfuromonas* spp. (39,50%)

Geoalkalibacter subterraneus et *Desulfuromonas* ssp. étaient majoritaires ou co-majoritaires dans les différents biofilms obtenus. Alors que ces deux espèces sont retrouvées en co-dominance lors de prélèvements aux maxima de production de densité de courant et après consommation totale du substrat dans les biofilms B8 et B0, seule Geoalkalibacter était dominante dans deux biofilms prélevés au maximum de production de courant dans les biofilms B7 et B9. Nous avons voulu vérifier le caractère aléatoire ou séquentiel de cette prédominance ou co-prédominance dans le biofilm.

Lors de la formation du biofilm électroactif, la mesure au cours du temps de la charge transférée à l'anode par le biofilm permet de suivre l'activité de la communauté microbienne en termes de transfert d'électrons. On peut assimiler la courbe de charge cumulée transférée à l'anode (Q en coulomb(C)) en fonction du temps à une courbe de croissance microbienne dont le taux de croissance est limité par la concentration en substrat. Nous nous sommes ici intéressés à la caractérisation des populations microbiennes au cours de cette croissance du biofilm.

Cette étude vise à prélever le biofilm électroactif aux différents stades de sa formation afin d'étudier sa dynamique de colonisation et de vérifier si les deux espèces majoritaires obtenues systématiquement, *Geoalkalibacter subterraneus* et *Desulfuromonas* ssp., s'implantent de façon séquentielle ou simultanée sur l'anode.

IV.3.2. MISE EN ŒUVRE

Afin de réaliser cette étude, nous avons utilisé un dispositif N-Stat. Le N-stat permet d'obtenir des biofilms électroactifs de compositions microbiennes identiques dans un même réacteur. Le réacteur comprenait 4 électrodes de graphite de dimension 2.5cm x 2.5cm, une grille de platine de dimension 5 cm x 5cm et une électrode de référence au calomel saturé (ECS). Chaque électrode de graphite était polarisée à +0.2V / ECS.

Des sédiments des salins de Saint-Martin à Gruissan ont été utilisés pour inoculer le réacteur N-Stat à 10% v/v dans un volume liquide de 500 mL contenant du tampon MES (50mM), le milieu Starkey modifié et du chlorure de sodium à 35 g_{NaCl}/L. Le réacteur était alimenté avec 1,2 g/L d'acétate. Le pH était fixé à 7 avec de la soude à 1M et la température était maintenue à 37°C avec un bain-marie.

La *Figure IV-10* présente le dispositif N-Stat. Les électrodes de graphites ont été utilisées dans ce dispositif pour obtenir des biofilms identiques dans un même milieu. Ces électrodes ont été successivement retirées du réacteur pour prélever les biofilms à différents stades de colonisation de l'anode Dynacol 1, Dynacol 2, Dynacol 3 et Dynacol 4.

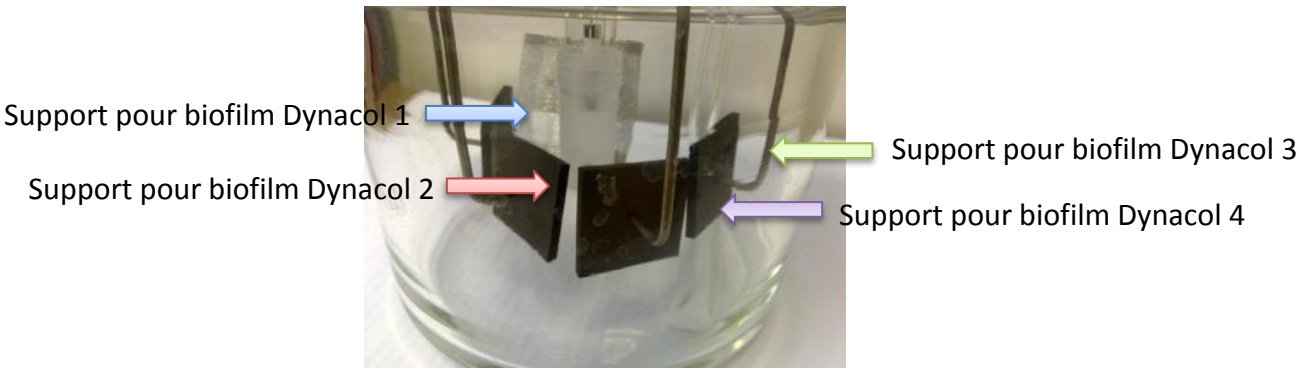


Figure IV-10 : Dispositif N-Stat utilisé contenant 4 électrodes de travail en graphite, 1 électrode de référence au calomel saturé et une contre électrode en platine (grille).

Les biofilms successifs utilisés pour analyser la composition de la communauté microbienne Dynacol 1 à 4 ont été prélevés successivement.

L'ADN total de la communauté du biofilm ainsi récupéré a été extrait pour des analyses d'empreintes moléculaires (CE-SSCP) et d'identification (pyroséquençage).

IV.3.3. RESULTATS ET DISCUSSION

La *Figure IV-11* présente les densités de courant obtenues pour chaque biofilm prélevé. Les courbes de chronoampérométrie des biofilms présentent une très bonne reproductibilité des productions de densité de courant dans le réacteur N-Stat utilisé. Des biofilms électroactifs présentant des densités de courant élevées ont été obtenus avec une densité de courant moyenne de 10.6±0 ,5 A/m². Cette valeur s'accorde avec nos résultats précédents obtenus dans les mêmes conditions où une densité de courant de 8,5 A/m² a été atteinte (dans la partie IV.1 intitulée « Sélection et identification de microorganismes électro-actifs »).

Les biofilms ont été prélevés dans la phase exponentielle de la courbe de charge (pour Dynacol 1 et Dynacol 2), en fin de phase exponentielle (Dynacol 3) et au plateau de production de charge, lorsque le substrat est consommé (Dynacol 4) (*Figure IV-11*).

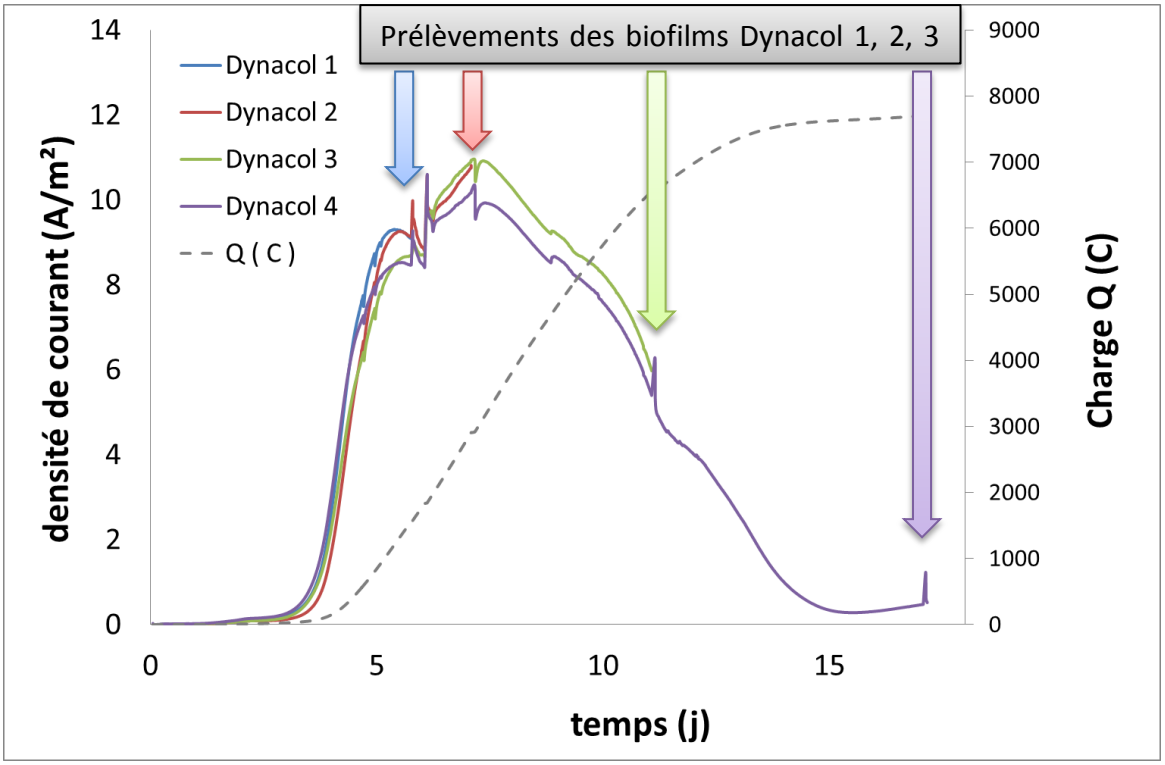


Figure IV-11 : Chronoampérométries réalisées sur les 4 électrodes Dynacol 1, 2, 3 et 4.

Chaque densité de courant est rapportée à la surface de colonisation totale d'une électrode de graphite. La courbe de charge Q en fonction du temps est reportée en pointillés pour une électrode (Dynacol 4).

Les profils de CE-SSCP des communautés microbiennes basées sur le gène ARNr16S des biofilms successivement prélevés sont présentés en *Figure IV-12*. Ces profils présentent une importante sélection microbienne, comme dans les études précédentes (parties IV.1 et IV.2) et dans la littérature avec certains systèmes électrochimiques à potentiel contrôlé (Torres et al., 2009; Harnisch et al., 2011; Miceli et al., 2012). Cette forte sélection peut être attribuée aux conditions expérimentales très spécifiques avec un accepteur d'électrons constitué par une électrode de graphite au potentiel imposé, un donneur d'électrons unique (acétate), un milieu minimum très simple, et aussi une régulation de la température à 37 °C, un pH fixé à 7 (Rabaey et al., 2010) .

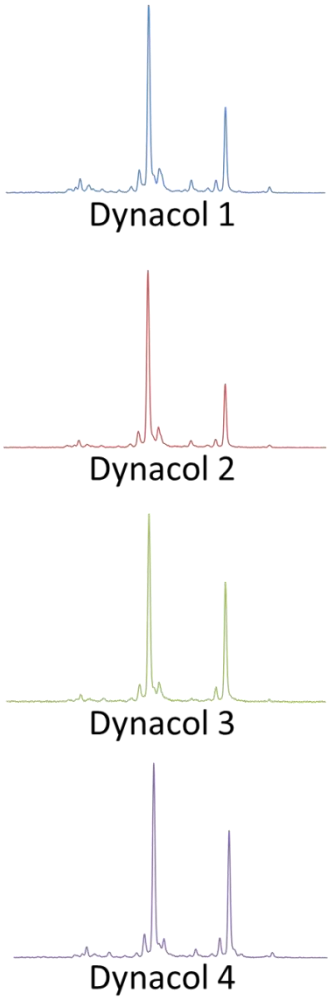


Figure IV-12 : Profils SSCP (des gènes d'ARN 16S) des communautés microbiennes des biofilms prélevés successivement (Dynacol 1, Dynacol 2, Dynacol 3 et Dynacol 4).

Les axes X et Y représentent respectivement la distance de migration de la séquence et l'intensité du pic.

D'autre part, les profils CE-SSCP obtenus au cours du temps sont superposables. Les espèces majoritaires sont donc soit identiques, soit très proches phylogénétiquement au niveau du gène ARN 16S.

De plus, la *Figure IV-13* représente les abondances relatives de chaque espèce dans les biofilms obtenus (Dynacol 1 à 4). Ces résultats obtenus par pyroséquençage s'accordent avec ceux des profils SSCP de la *Figure IV-12* et montrent une importante sélection microbienne au sein du biofilm. La composition des biofilms montre la présence de *Geoalkalibacter subterraneus* et *Desulfuromonas* sp. en co-dominance dès les premières étapes d'attachement sur l'électrode. Ces deux espèces, qui ont été précédemment retrouvées en cultures mixtes (parties IV.1 et IV.2), ont montré de bonnes capacités à transmettre leurs électrons à une anode lorsqu'elles sont testées en culture pure (Bond et al., 2002; Badalamenti et al., 2013; Carmona-Martinez et al., 2013).

Nos résultats sont en accord avec une étude de Patil et al. (2011) dans laquelle des biofilms successifs issus d'essais en mode fed-batch présentaient une forte sélection des populations microbiennes au cours du temps dans 3 séries d'expériences à pH 6, 7 et 9. Cependant, les résultats de Patil et al. (2011) montrent une dynamique de la structure de communauté microbienne au cours des fed-batchs à pH 6 et 9 qui n'apparaissent pas à pH 7. Il est intéressant de noter que les résultats présentés en *Figure IV-13* confirment cette sélection à pH 7. Le changement de structure de communauté microbienne à pH 6 et pH9 dans l'étude de Patil et al. (2011) pourrait être dû à une carence des microorganismes spécifiquement sélectionnés à ces valeurs de pH par rapport à des éléments présents dans l'inoculum de départ, mais aussi à une sélection plus diverse de microorganismes moins électroactifs qu'à pH 7. En effet, la densité de courant produite est plus élevée à pH 7 qu'aux autres pH testés par Patil et al. (2011).

La proportion de *Malonomonas rubra*, qui compose 4% de la distribution phylogénétique dans le premier biofilm (Dynacol 1), diminue au cours de la colonisation du biofilm pour ne constituer que moins de 1% en abondance relative de la population microbienne. Une espèce proche de *Malonomonas rubra* (avec 95 % de similarité sur l'ARN 16S) a précédemment été isolée à partir de biofilms électroactifs issus de sédiments marins (en pile à combustible de laboratoire) et de sédiments de salins (en pile à combustible *in situ*) dans une étude de Holmes et al. (2004). Holmes et al. (2004) ont également montré la capacité de *Malonomonas rubra* à réduire des oxydes de fer (III) comme les DMRB qui composent

souvent les biofilms électroactifs. Il a par ailleurs été montré que *Malonomonas rubra* est très proche phylogénétiquement de *Desulfuromonas acetoxidans* (92% de similarité entre les séquences d'ARNr 16S) (Kolb et al., 1998). De plus, elle possède des cytochromes de type c similaires à ceux de la sulfato-réduction chez d'autres organismes (Kolb et al., 1998). La similarité avec une autre espèce électroactive et la présence de cytochromes c pouvant assurer le transfert d'électron vers un accepteur externe s'accorde avec l'enrichissement de *Malonomonas rubra* sur anode retrouvé ici. La diminution de la proportion de *Malonomonas rubra* au cours du temps au profit de *Geoalkalibacter subterraneus* et *Desulfuromonas* sp. peut être due aux conditions imposées par le système. Notamment, *Malonomonas* présente une température optimale de croissance entre 28 et 30 °C (Vandieken et al., 2006) alors que la température ici était maintenue à 37°C. La présence de *Malomonas rubra* suggère donc une étude plus approfondie de cette espèce en culture pure pour vérifier ses capacités en termes d'électroactivité et en co-culture avec d'autres espèces comme *Geoalkalibacter subterraneus* ou *Desulfuromonas* sp. pour étudier l'influence de plusieurs taux d'inoculation sur les performances du système.

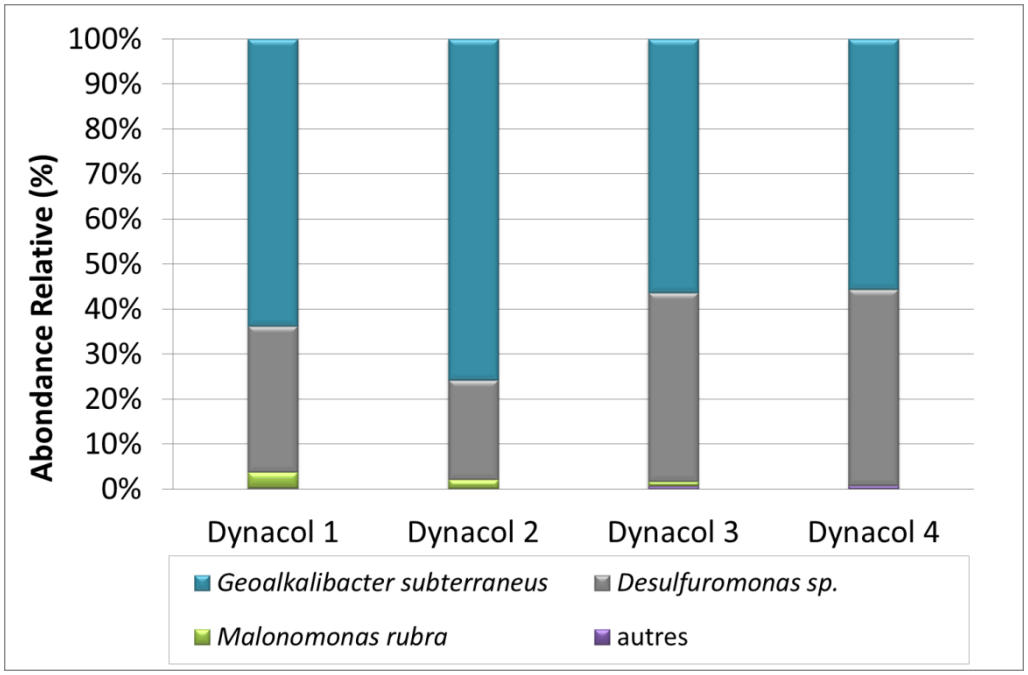


Figure IV-13 : Distribution taxonomique des bactéries phylogénétiquement les plus proches identifiées dans les différents biofilms collectés au cours de la colonisation de l'électrode.

L'abondance relative est définie par rapport au nombre de séquences affiliées à un taxon divisé par le nombre total de séquences par échantillon. Les espèces constituant moins de 1% de la population microbienne du biofilm ont été placées dans le groupe « autres ».

Finalement cette expérience a montré que, dans des conditions contrôlées (pH 7, 37°C, 35 g_{NaCl}/L) et en utilisant des sédiments salins comme inoculum, une forte sélection des espèces électroactives *Geoalkalibacter subterraneus* et *Desulfuromonas* sp. en co-dominance se produisait dès les premiers jours de formation du biofilm. La composition microbienne reste la même au cours de l'expérience et le prélèvement du biofilm pour la caractérisation microbienne peut donc être fait à différents stades tout en restant représentatif.

Il serait intéressant de réaliser cette même expérience dans d'autres conditions de pH pour voir si les observations de Patil et al. (2011) en fed-batch à pH 6 et pH9 peuvent être faites en batch. D'autre part, il pourrait aussi être intéressant de tester d'autres sources d'inocula puisque la composition microbienne du biofilm en dépend.

IV.4. CONCLUSION SUR LE CHAPITRE IV

L'étude des biofilms électroactifs menée dans ce chapitre a permis, dans un premier temps, d'identifier de nouvelles sources de consortia bactériens électroactifs : les sédiments salins qui ont permis d'obtenir des biofilms électroactifs performants en conditions salines. Cependant, le biofilm n'a pas présenté de bonnes capacités d'adaptation à un pH plus acide (pH 5,5), classiquement utilisé en fermentation pour la production d'hydrogène.

Ensuite, cette étude a permis de caractériser cet inoculum et d'identifier les espèces majoritaires qui le composent et notamment *Geoalkalibacter subterraneus* qui est nouvellement définie comme électroactive. Les biofilms obtenus ont atteint jusqu'à 8.5 A/m² (IV.1) en réacteur à 3 électrode et 10.6±0 ,5 A/m² en réacteur N-Stat à 6 électrodes (IV.3), ce qui est cohérents avec les études antérieures trouvées dans le littérature notamment avec *Geoalkalibacter subterraneus* (Miceli et al., 2012).

Une méthode d'enrichissement a ensuite été étudiée pour enrichir les DMRB (Dissimilatory Metal-Reducing Bacteria) à partir de l'inoculum de départ en partant de l'hypothèse que la capacité des DMRB à transférer des électrons à des oxydes de fer (III) était transposable à un échange d'électrons avec l'anode. L'aspect microbien de la sélection dans un milieu enrichi en fer (III) a été mis en regard avec les performances électroactives du biofilm. Cette méthode pourrait permettre de supprimer les bactéries planctoniques et les accepteurs d'électrons, initialement présent dans les sédiments, qui sont en compétition pour le substrat et avec l'électrode.

L'étude comparative de la sélection microbienne au cours des deux stratégies d'enrichissement (sur anode et sur Fe(III)) a montré une divergence de sélection microbienne qui peut être associée à une baisse des performances électroactives du biofilm après plusieurs étapes de pré-enrichissement sur Fe(III). Cependant, ici, une seule étape d'enrichissement a été nécessaire pour améliorer ces performances. Les résultats de notre étude ont montré que la méthode de pré-enrichissement sur Fe(III) doit être réalisée en très peu d'étapes pour ne pas obtenir une communauté microbienne trop spécialisée pour la respiration sur Fe(III). Cette méthode présente un intérêt majeur dans les stratégies d'inoculation de BES car elle permet de s'affranchir de la conduite préalable de réacteur, elle

fournit un inoculum liquide plus simple à manipuler qu'un biofilm et elle permet d'éviter la perte éventuelle d'activité des bactéries électroactive par repiquages successifs.

La forte sélection de ces espèces électroactives et le caractère aléatoire de leur dominance ou codominance dans le biofilm ont mené à l'étude de la dynamique de colonisation de ces espèces sur l'électrode afin d'établir si ces deux espèces majoritaires obtenues systématiquement sur anode dans les deux premières parties s'implantent de façon séquentielle. Cette étude de la dynamique de colonisation des électrodes a permis de confirmer le caractère précoce de la dominance ou codominance de espèces électroactives *Geoalkalibacter subterraneus* et *Desulfuromonas* sp. dès l'attachement du biofilm sur l'électrode.

Finalement la forte sélection des espèces électroactives sur l'électrode avec une ou deux espèce(s) majoritaire(s) dans le biofilm pourrait être attribuée au caractère très sélectif du milieu de par sa composition, ses paramètres opératoires (pH 7, 37°C, 35 g_{NaCl}/L) et du dispositif expérimental (avec une anode à potentiel contrôlé).

Dans ce chapitre, nous avons observé une forte sélection dans chaque biofilm électroactif, et donc une forte simplification de la diversité microbienne. Un biofilm présentant une communauté microbienne diverse est pourtant plus résistant face aux perturbations exogènes biotiques ou abiotiques. Suite à ce travail, nous nous sommes donc intéressés à l'influence de l'ajout des espèces exogènes sélectionnées en fermentation dans le Chapitre III sur le biofilm électroactif en termes de performances électroactives et de perturbation des communautés microbiennes qui le composent.

CHAPITRE V. 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*).

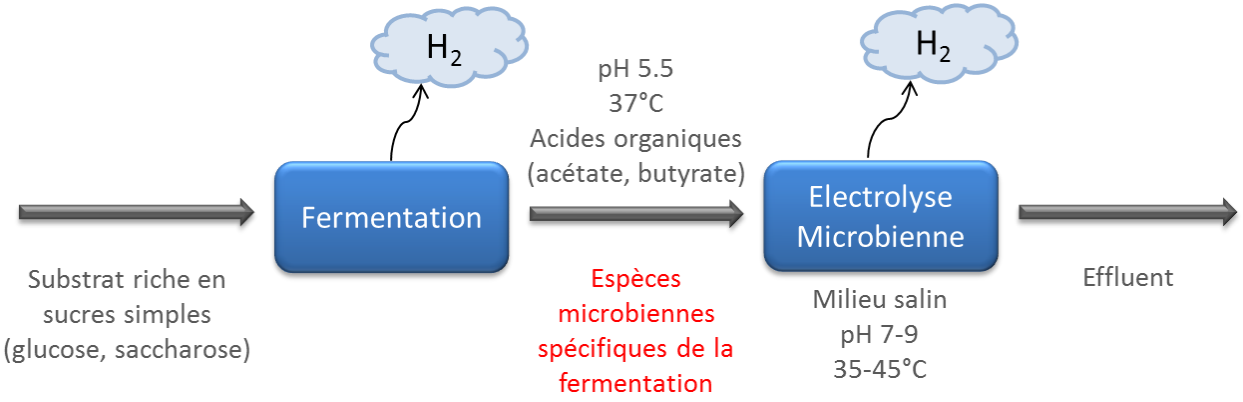


Figure V-1 : Paramètres spécifiques liés au procédé de fermentation et d'électrolyse microbienne dans le cadre du couplage.

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 :

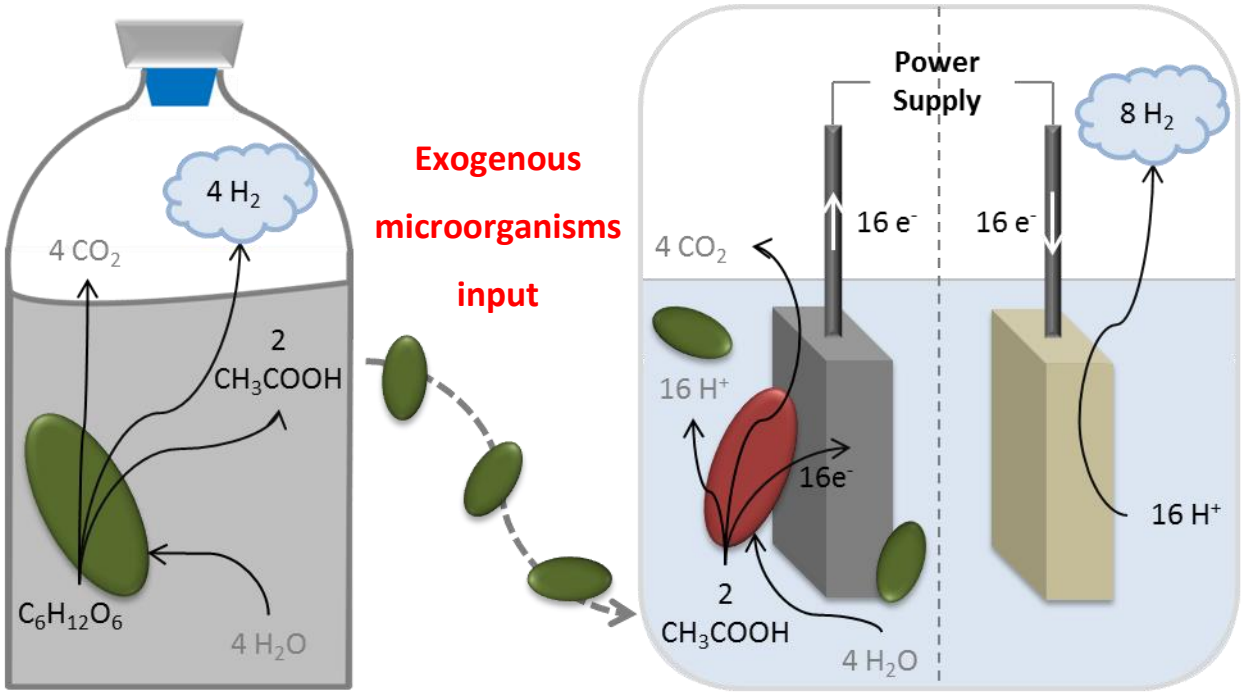
Biohydrogen production is of increasing interest in a context of fossil fuel increasing deficiency. Dark fermentation process is limited to a theoretical conversion yield of 4 $mol_{H2}/mol_{Glucose}$ with concomitant production of organic acids such as acetate and butyrate. Microbial electrolysis completely 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).

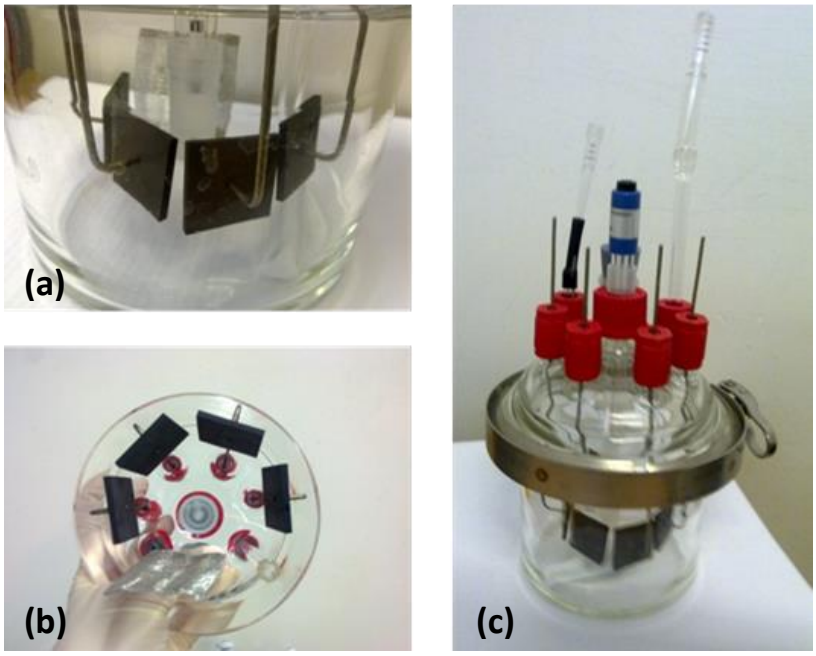


Figure V-2 : N-Stat arrangement to grow electroactive biofilms.

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.

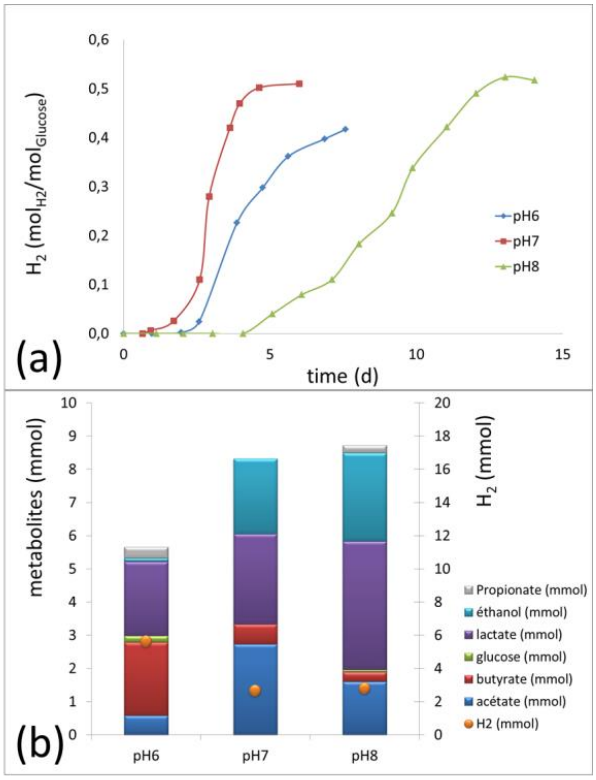


Figure V-3 : Hydrogen production of biomass used as exogenous bacteria on biofilm at the maximum hydrogen production point.

(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)

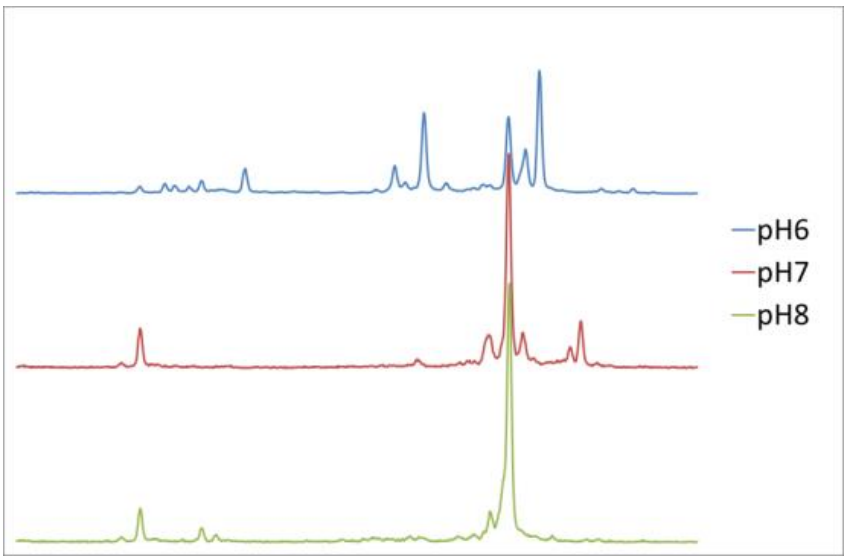


Figure V-4 : CE-SSCP profiles of biomass based on 16S rRNA gene fragments retrieved from fermentation batches at pH 6, pH 7 and pH 8.

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).

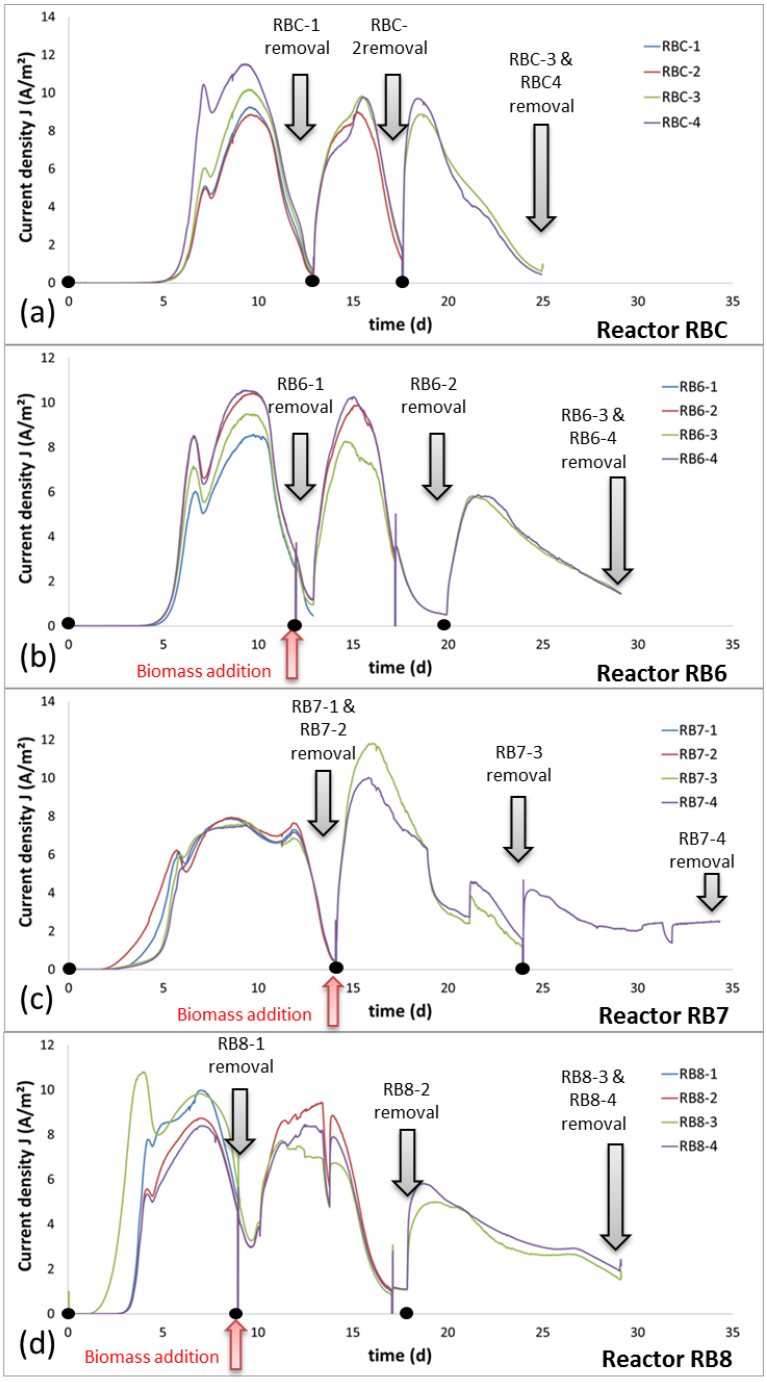


Figure V-5 : Chronoamperometry in the different systems:

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.

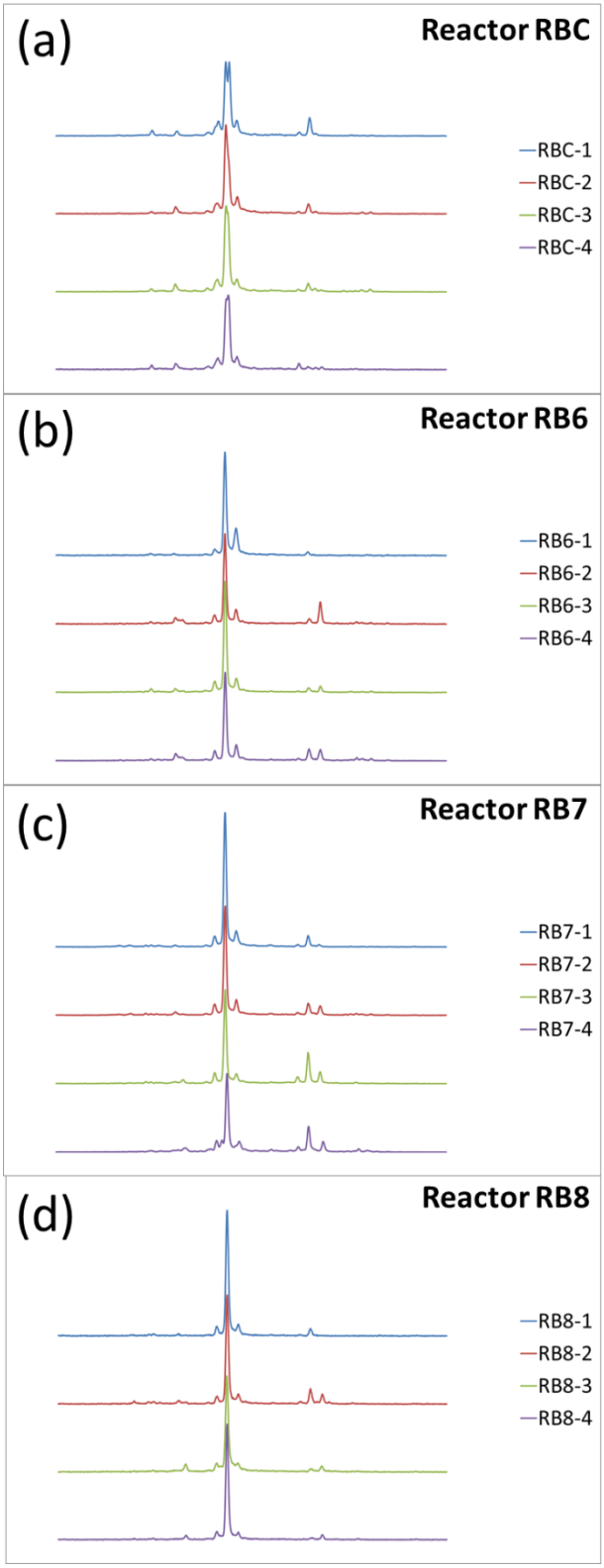


Figure V-6 : CE-SSCP profiles of biomass based on 16S rRNA gene fragments retrieved from biofilms in the 4 different reactors:

(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.

V.3. DISCUSSION ET CONCLUSION

Ce travail portait sur la détermination de l'impact de l'ajout d'espèces exogènes issues de la fermentation sur le biofilm électroactif, tant sur ses propriétés électroactives que sur sa structure de communauté microbienne. Les espèces exogènes et le biofilm électroactif ont été choisis en fonction des études préalables réalisées dans le cadre de cette thèse. Trois type de biomasses fermentaires différentes par leur pH de croissance ont été utilisées : pH 6, 7 ou 8 correspondant respectivement aux pH de la fermentation pour produire du bioH2 dans la littérature (Guo et al., 2010), de l'électrolyse microbienne (Logan, 2012) et au pH naturel de l'inoculum également utilisé lors de la fermentation réalisée dans le Chapitre III (Pierra et al., 2013). Le biofilm électroactif a quant à lui été enrichi sur anode à partir de sédiments des salins de Saint Martin à Gruissan. Comme dans les chapitres précédents, tous les milieux (fermentation et réacteur bioélectrochimique) étaient salins avec une concentration en chlorure de sodium de 35 g_{NaCl}/L. Tous les réacteurs bio-électrochimiques ont été conduits à pH 7.

Les densités de courants obtenues dans cette étude sont élevées par rapport à la littérature dans des conditions similaires de salinité (Erable et al., 2009; Miceli et al., 2012) (9.8±0.8 A/m² dans RB6, 10,8±1,3 dans RB7, 9.6±0.8 A/m² dans RB8, 9,3±0,1 A/m² dans RBC au premier cycle). Les communautés microbiennes développées sur le biofilm étaient très simplifiées avec un profil SSCP présentant un ou deux phylotypes principaux. L'ajout d'espèces exogènes n'a pas montré de modification significative de structure de communauté microbienne pour toutes les conditions de fermentation testées, ce qui laisse supposer que la forte sélection des espèces électroactives sur l'anode et l'efficacité de ce biofilm vont de pair avec la robustesse du biofilm.

De plus, l'augmentation de l'épaisseur du biofilm constatée visuellement lors des prélèvements successifs dans les réacteurs RB6, RB7 et RB8, (cf. exemple en Figure Annexe-9 de annexe 2) ayant reçu respectivement la biomasse exogène issue de fermenteurs à pH 6, pH 7 et pH 8 ainsi que le maintien de la quantité de bactéries dans ces biofilms laisse supposer que la communauté microbienne qui compose le biofilm ait développé un réseau d'EPS (Extracellular Polymeric Substances) pour se protéger d'une éventuelle colonisation du biofilm par les espèces exogènes (Burmølle et al., 2006).

L'augmentation de l'épaisseur du biofilm et la séquestration du substrat dans la matrice d'EPS pourrait expliquer le maintien d'une densité de courant plus faible sur une plus longue période en 3ème cycle de batch (*Figure V-5*) et un maintien du rendement faradique (*Tableau V-1*) (Freguia et al., 2007).

Enfin en perspectives, il serait intéressant de réitérer cette expérience en continu et non en batch successifs. En effet, la présence de méthane dans le ciel gazeux des réacteurs bioélectrochimique laisse supposer un développement de méthanogènes consommateurs d'hydrogène et d'acétate(Lee et al., 2009). Ces méthanogènes pourraient donc à la fois affecter directement la production d'hydrogène mais aussi être en compétition avec le biofilm électroactif pour la consommation de substrat (acétate). La conduite de réacteur en continue permettrait un lessivage de ces méthanogènes et éviterait qu'elles s'établissent dans le compartiment anodique du fait de leur temps de doublement plus long.

Pour l'heure en termes d'application sur le procédé de couplage, les résultats en termes de rendement faradique et donc de conversion du substrat en électrons montre une activité maintenue du biofilm électroactif. La réalisation du couplage en cellule d'électrolyse microbienne avec séparation des compartiments anodique et cathodique par une membrane permettrait d'éviter l'oxydation de l'hydrogène produit à la cathode par l'anode et d'obtenir une tendance plus juste de la réelle efficacité électroactive du biofilm.

Nota : Les pyroséquençages des communautés microbiennes des réacteurs RB6 et RB7 ainsi que des communautés fermentaires issues des fermenteurs batch à pH 6 et pH 7 est en cours de réalisation.

CONCLUSIONS ET PERSPECTIVES

L'objet de cette thèse était d'analyser les liens entre la structure des communautés microbiennes en MEC et en fermentation, les individus qui les composent et les fonctions macroscopiques (propriétés électroactives du biofilm, potentiel de production d'hydrogène) qui leur sont associées dans des conditions permettant de réaliser le couplage des deux procédés.

Dans un premier temps, la fermentation en condition saline (3-75 g_{NaCl}/L) favorable au transport de charges dans l'électrolyte de la MEC, a été étudiée à pH 6, favorable à la production d'hydrogène, et pH 8, pH de l'inoculum salin dans son environnement. A pH 8 comme pH 6, la faisabilité de la production de biohydrogène par fermentation a été démontrée en lien avec l'inhibition de la consommation de l'hydrogène produit. Les essais en batch à pH 8 ont également révélé une très forte prédominance d'une nouvelle souche de *Vibrionaceae* pour les concentrations en sel les plus élevées (58-75 g_{NaCl}/L). A pH 6, des espèces proches des clostridies produiraient de l'hydrogène jusqu'à 41 g_{NaCl}/L avec de meilleurs rendement qu'à 3 g_{NaCl}/L mais une vitesse de production maximale plus faible. Cette étude a montré, contrairement aux études précédentes, une production d'hydrogène supérieure aux concentrations en sel marines à pH 6 (entre 12 et 48 g_{NaCL}/L) et croissantes à pH 8 (jusqu'à 70g_{NaCl}/L). Ces résultats sont vraisemblablement dus à l'utilisation de sédiments salins comme inoculum, contenant donc des microorganismes adaptés à ces conditions salines.

Dans un deuxième temps, la sélection microbienne et les performances électroactives de biofilms (densité de courant produite et rendement faradique) ont été étudiées dans des conditions compatibles avec la fermentation et en condition saline pour augmenter la conductivité en MEC, en utilisant également un inoculum salin. Des conditions de pH de 5,5 et 7 ont été testées ainsi que l'alimentation du biofilm avec de l'acétate seul ou un mélange d'acétate et de butyrate. Ces essais ont permis la sélection de deux espèces microbiennes dominantes dans tous les biofilms anodiques (*Geoalkalibacter subterraneus* et *Desulfuromonas acetoxidans*). Ces espèces présentent des propriétés électroactives prometteuses avec des densités de courant produites jusqu'à 8,5 A/m². Les performances atteintes par le biofilm à pH 5,5 et avec ajout d'un mélange d'acides organiques étaient moindres qu'à pH 7 alimenté avec de l'acétate seul. Dans le cadre d'un couplage de la fermentation et de l'électrolyse microbienne, les conditions de fermentation à pH 8 testées dans la première partie apparaissent particulièrement intéressantes du fait de la conduite difficile du biofilm électroactif à pH bas, même en milieu salin.

En parallèle, une méthode d'enrichissement a été étudiée afin de sélectionner ces espèces sur leur capacité à transférer leurs électrons à des oxydes de Fer(III) à partir d'une source d'inoculum naturelle. Trois étapes d'enrichissements successives sur Fer(III) ont été réalisées. Une divergence des communautés microbiennes sélectionnées dans les enrichissements liquides et sur l'anode a été observée. Celle-ci peut être corrélée à une diminution des performances électroactives du biofilm après deux ou trois étapes d'enrichissement sur Fe(III). Toutefois, l'utilisation de la première étape d'enrichissement liquide comme inoculum pour former un biofilm a permis d'augmenter la conversion d'acétate en électrons transférés à l'anode (le rendement faradique) de 30±4% à 99±8% par rapport à un biofilm directement inoculé avec les sédiments de départ. Ces résultats permettent d'expliquer la divergence des opinions rapportées dans la littérature sur l'efficacité de cette méthode pour sélectionner des ARB. En effet, les différentes études précédentes ont été réalisées avec un nombre variable de cycles d'enrichissements qui impactait les performances électroactives du biofilm. Suite à cette étude, il a été suggéré d'optimiser l'utilisation de cette méthode de pré-sélection des ARB en ne réalisant qu'un unique cycle d'enrichissement sur Fe(III) afin de ne pas spécialiser la communauté microbienne, par repiquages successifs, pour une respiration sur Fe(III).

De plus, deux espèces électroactives (*Geoalkalibacter subterraneus* et *Desulfuromonas* spp.) ont été retrouvées en dominance ou codominance dans les biofilms obtenus dans les expériences réalisées dans les deux premières parties de cette thèse. Ceci a mené à une étude de leur dynamique de colonisation sur l'électrode afin d'établir si ces deux espèces majoritaires obtenues systématiquement sur anode s'implantent de façon séquentielle. Au contraire, nous avons observé le caractère précoce de la dominance ou codominance de *Geoalkalibacter subterraneus* et *Desulfuromonas* spp. qui est établie dès le début de la phase exponentielle du transfert d'électrons du biofilm vers l'électrode, sans évolution de la communauté microbienne du biofilm par la suite. La sélection très marquée de ces espèces électroactives était vraisemblablement due au caractère très sélectif du milieu et aux paramètres opératoires très contrôlés (pH7, 37°C, 35 g_{NaCl}/L, anode à potentiel contrôlé).

Enfin l'effet de l'ajout d'espèces exogènes issues de la fermentation en milieu salin sur l'écologie du biofilm électroactif a été étudié. Trois types de biomasses fermentaires ont été ajoutés au milieu contenant le biofilm électroactif formé au préalable. Ces biomasses étaient issues de réacteurs batch conduits à différentes valeurs de pH correspondants aux pH de la fermentation dans la littérature (pH 6), de l'électrolyse microbienne (pH 7) et de l'inoculum et de la fermentation réalisée dans la première partie de la thèse (pH 8). Cette expérience a montré que l'ajout des espèces exogènes sélectionnées en fermentation n'a pas d'impact significatif sur la structure de communauté microbienne du biofilm. Ceci suggère que malgré la forte sélection d'espèces électroactives et l'efficacité de ces biofilms, ils présentent une certaine robustesse vis-à-vis de perturbations biotiques. Cependant une augmentation de l'épaisseur du biofilm constatée visuellement dans les réacteurs contaminés avec des espèces exogènes et non dans le réacteur témoin suggère une production d'EPS (Extracellular Polymeric Substances) par le biofilm pour se prémunir d'une éventuelle invasion par ces espèces exogènes. Ceci pourrait expliquer la diminution de la densité de courant observée dans ces biofilms : le transfert de substrat y serait ralenti en raison de cet épaississement, se traduisant par une diminution de la vitesse de transfert d'électrons vers l'anode.

L'ensemble des expérimentations présentées dans cette thèse ont permis de mettre en lien les structures de communautés microbiennes en fermentation et en MEC avec leurs performances en termes de production d'hydrogène et d'électroactivité dans des conditions permettant de réaliser le couplage des deux procédés. Le même inoculum salin anaérobie et présentant une importante diversité a été utilisé avec succès en fermentation et en BES. Issu d'un environnement anaérobie riche en matière organique et en sel, soumis à des conditionnements et des pressions de sélections différents, il a fait émerger des communautés microbiennes performantes en fermentation comme en BES. Cette origine commune des différentes communautés microbiennes pourrait expliquer la non-contamination du biofilm électroactif en BES par les espèces issues des effluents de fermentation, sélectionnées à partir du même inoculum de départ. Ce travail souligne donc l'importance du choix de l'inoculum, de la stratégie de son conditionnement et de sa mise en œuvre sur le fonctionnement de bioprocédés en cultures mixtes.

Les résultats de cette thèse offres d'intéressantes perspectives de recherche, notamment:

- Les deux souches systématiquement retrouvées dans les biofilms électroactif de cette étude comme dominantes ou co-dominantes (*Geoalkalibacter subterraneus* et *Desulfuromonas acetoxidans*) peuvent être cultivées en culture pure et en co-culture à différents ratios d'inoculation afin d'étudier leurs éventuelles interactions et les conséquences sur l'électroactivité du biofilm. Les conclusions de cette étude pourraient permettre d'envisager une démarche d'ingénierie écologique consistant à ajouter ces souches à des inocula naturels pour améliorer les performances du système.

- D'autre part, la stratégie d'inoculation après enrichissement sur Fe(III) n'a été testée que sur un seul inoculum (sédiments). D'après les résultats de sélection d'un inoculum obtenus précédemment, ces sédiments constituaient une bonne source de bactéries électroactives. Cette méthode d'enrichissement pourrait être testée sur des sources d'inoculum différentes et qui ne présentent pas *a priori* un potentiel aussi élevé pour la formation de biofilm électroactifs.

- Dans cette étude, un dispositif à 3 électrodes a été utilisé pour étudier le biofilm électroactif. Ce dispositif permettait de fixer et de contrôler le potentiel de l'anode. Au terme de ce travail, il est suggéré que la sélection microbienne opérée dans le biofilm soit due à des paramètres opératoires très contrôlés, notamment ce potentiel fixe à l'anode. En électrolyse microbienne, sans électrode de référence, le potentiel anodique peut varier. Il serait donc intéressant de voir si dans une configuration d'électrolyse microbienne, malgré une stabilité moindre du potentiel anodique, une telle sélection est obtenue.

- Dans l'optique du développement d'un électrolyseur microbien, afin de découpler clairement les phénomènes se déroulant à l'anode et à la cathode, il serait judicieux de séparer les deux compartiments par une membrane. Il serait ainsi possible d'établir précisément les rendements faradiques à l'anode et de production d'hydrogène à la cathode. En effet, dans notre dispositif expérimental, une partie de l'hydrogène produit à la cathode pouvait être

oxydée à l'anode et fausser ainsi les calculs de ces rendements, à la hausse pour le premier, à la baisse pour le second.

- La présence de méthane dans le ciel gazeux des réacteurs bioélectrochimiques laisse supposer un développement de méthanogènes consommateurs d'hydrogène et d'acétate. Ces méthanogènes pourraient donc à la fois affecter directement la production d'hydrogène mais aussi être en compétition avec le biofilm électroactif pour la consommation de substrat (acétate). Il serait intéressant de réitérer l'expérience d'ajout d'espèces exogènes sur un biofilm électroactif en continu et non en batch successifs. La conduite de réacteur en continu permettrait un lessivage de ces méthanogènes et éviterait qu'elles s'établissent dans le compartiment anodique du fait de leur faible taux de croissance.

- Enfin, il pourra être envisagé d'intégrer la technologie des MEC dans une filière de production de biohydrogène par fermentation en réacteurs pilotes et ce en configuration en série ou en couplage intégré. Les interactions des microflores spécifiques de chaque procédé pourront être mises en regard avec les performances électroactives du biofilm et de production d'hydrogène des deux procédés. L'évolution au cours du temps de ces communautés microbiennes en fonction des paramètres opératoires (pH, température, temps de séjour) pourra être étudiée. De plus, suite aux résultats apportés par cette thèse, la conduite de fermenteurs en milieu salin pourra être envisagée dans le cadre du couplage afin d'augmenter la conductivité dans la MEC. Le traitement d'effluents salins spécifiques tels que les effluents issus d'industries agroalimentaires (salaisons), de tanneries ou de plateforme de traitement des déchets (lixiviats) pourrait ainsi être couplé à la production d'hydrogène par couplage de fermentation et d'électrolyse microbienne.

REFERENCES BIBLIOGRAPHIQUES

APHA, AWWA, WPCF, 1995. Standard methods for the examination of water and wastewater, 19th edition, i. ed. Washington DC.

Abrevaya, X.C., Sacco, N., Mauas, P.J.D., Cortón, E., 2011. Archaea-based microbial fuel cell operating at high ionic strength conditions. Extremophiles 15, 633–642.

Aceves-Lara, C.A., Latrille, E., Buffiere, P., Bernet, N., Steyer, J.-P., 2008. Experimental determination by principal component analysis of a reaction pathway of biohydrogen production by anaerobic fermentation. Chemical Engineering and Processing 47, 1968–1975.

Adams, M.W.W., Mortenson, L.E., 1984. The Physical and Catalytic Properties of Hydrogenase I1 of *Clostridium pasteurianum*. The Journal of Biological Chemistry 259, 7045–7055.

Aelterman, P., Rabaey, K., Verstraete, W., 2006. Continuous Electricity Generation at High Voltages and Currents Using Stacked Microbial Fuel Cells. Environmental Science & Technology 40, 3388–3394.

Alleau, T., 2007. L'Hydrogène, énergie du futur ?, EDP Sciences

Alshiyab, H., Kalil, M., Hamid, A., Wan Yusoff, W., 2008. Effect of salts addition on hydrogen production by *C. acetobutylicum*. Pakistan Journal of Biological Sciences 11, 2193–2200.

Amin, S. a, Green, D.H., Gärdes, A., Romano, A., Trimble, L., Carrano, C.J., 2012. Siderophore-mediated iron uptake in two clades of *Marinobacter* spp. associated with phytoplankton: the role of light. Biometals 25, 181–192.

Arends, J.B. a, Verstraete, W., 2012. 100 Years of Microbial Electricity Production: Three Concepts for the Future. Microbial Biotechnology 5, 333–346.

Argun, H., Kargi, F., Kapdan, I., Oztekin, R., 2008. Batch dark fermentation of powdered wheat starch to hydrogen gas: Effects of the initial substrate and biomass concentrations. International Journal of Hydrogen Energy 33, 1813–1819.

Asghari, I., Mousavi, S.M., Amiri, F., Tavassoli, S., 2013. Bioleaching of spent refinery catalysts: A review. Journal of Industrial and Engineering Chemistry 19, 1069–1081.

Association Française de l'hydrogène, n.d. Association Française de l'hydrogène [WWW Document]. URL http://www.afhypac.org/fr/accueil

Atif, a, Fakhrulrazi, A., Ngan, M., Morimoto, M., Iyuke, S., Veziroglu, N., 2005. Fed batch production of hydrogen from palm oil mill effluent using anaerobic microflora. International Journal of Hydrogen Energy 30, 1393–1397.

Badalamenti, J.P., Krajmalnik-brown, R., Torres, C.I., 2013. Generation of high current densities by pure cultures of anode- respiring *Geoalkalibacter* spp . under alkaline and saline conditions. mBio 4, 1–8.

Bennetto, H.P., Dew, M.E., Stirling, J.L., 1981. Rates of reduction of phenothiazine "redox" dyes by *E.coli*. Chemistry and Industry 776–778.

Biffinger, J.C., Pietron, J., Bretschger, O., Nadeau, L.J., Johnson, G.R., Williams, C.C., Nealson, K.H., Ringeisen, B.R., 2008. Biosensors and Bioelectronics The influence of acidity on microbial fuel cells containing *Shewanella oneidensis*. Biosensors and Bioelectronics 24, 900–905.

Bond, D.R., Holmes, D.E., Tender, L.M., Lovley, D.R., 2002. Electrode-reducing microorganisms that harvest energy from marine sediments. Science 295, 483–485.

Bond, D.R., Lovley, D.R., 2003. Electricity production by *Geobacter sulfurreducens* attached to electrodes. Applied and Environmental Microbiology 69, 1548–1555.

Bond, D.R., Lovley, D.R., 2005. Evidence for involvement of an electron shuttle in electricity generation by *Geothrix fermentans*. Applied and Environmental Microbiology 71, 2186–2189.

Braun, F., 2012. Impact des communautés microbiennes sur la dynamique de dégradation de micropolluants organiques au sein d ’écosystèmes épuratoires et sur l ’expression de leur toxicité. Thèse de l’Université de Montpellier 2.

Braun, F., Hamelin, J., Gévaudan, G., Patureau, D., 2011. Development and application of an enzymatic and cell flotation treatment for the recovery of viable microbial cells from environmental matrices such as anaerobic sludge. Applied and Environmental Microbiology 77, 8487–8493.

Brown, S.D., Begemann, M.B., Mormile, M.R., Wall, J.D., Han, C.S., Goodwin, L.A., Pitluck, S., Land, M.L., Hauser, L.J., Elias, D.A., 2011. Complete genome sequence of the haloalkaliphilic , hydrogen-producing bacterium *Halanaerobium hydrogeniformans*. Journal of Bacteriology 193, 3682–3683.

Burmølle, M., Webb, J.S., Rao, D., Hansen, L.H., Sørensen, S.J., Kjelleberg, S., 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Applied and Environmental Microbiology 72, 3916–3923.

Butler, J.E., Young, N.D., Lovley, D.R., 2010. Evolution of electron transfer out of the cell : comparative genomics of six *Geobacter* genomes. BMC genomics 11, 1–40.

Caccavo, F., Lonergan, D.J., Lovley, D.R., Davis, M., Stolz, J.F., McInerneyl, M.J., 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Applied and Environmental Microbiology 60, 3752–3759.

Call, D., Logan, B., 2008. Hydrogen production in a single chamber microbial electrolysis cell lacking a membrane. Environmental Science & Technology 42, 3401–3406.

Call, D., Wagner, R., Logan, B., 2009. Hydrogen production by *Geobacter* species and a mixed consortium in a microbial electrolysis cell. Applied and Environmental Microbiology 75, 7579–7587.

Carmona-Martinez, A. a, Harnisch, F., Fitzgerald, L. a, Biffinger, J.C., Ringeisen, B.R., Schröder, U., 2011. Cyclic voltammetric analysis of the electron transfer of Shewanella oneidensis MR-1 and nanofilament and cytochrome knock-out mutants. Bioelectrochemistry 81, 74–80.

Carmona-Martínez, A., Pierra, M., Trably, E., Bernet, N., 2013. Electrochemical and microscopic characterization of the novel anode respiring bacterium *Geoalkalibacter subterraneus* under saline

conditions: insights into its electron transfer mechanism and biofilm formation. Physical Chemistry Chemical Physics. DOI: 10.1039/c3cp54045f

Carmona-Martínez, A. a, Harnisch, F., Kuhlicke, U., Neu, T.R., Schröder, U., 2012. Electron transfer and biofilm formation of *Shewanella putrefaciens* as function of anode potential. Bioelectrochemistry.

Catal, T., Li, K., Bermek, H., Liu, H., 2008. Electricity production from twelve monosaccharides using microbial fuel cells. Journal of Power Sources 175, 196–200.

Chae, K.-J., Choi, M.-J., Lee, J.-W., Kim, K.-Y., Kim, I.S., 2009. Effect of different substrates on the performance, bacterial diversity, and bacterial viability in microbial fuel cells. Bioresource Technology 100, 3518–3525.

Chae, K.-J.K., Choi, M.-J.M., Lee, J., Ajayi, F.F.F., Kim, I.I.S., 2008. Biohydrogen production via biocatalyzed electrolysis in acetate-fed bioelectrochemical cells and microbial community analysis. International Journal of Hydrogen Energy 33, 5184–5192.

Chaudhuri, S.K., Lovley, D.R., 2003. Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. Nature Biotechnology 21, 1229–1232.

Chen, S., He, G., Liu, Q., Harnisch, F., Zhou, Y., Chen, Y., Hanif, M., Wang, S., Peng, X., Hou, H., Schröder, U., 2012. Layered corrugated electrode macrostructures boost microbial bioelectrocatalysis. Energy & Environmental Science 5, 1–6.

Chen, S., Hou, H., Harnisch, F., Patil, S. a., Carmona-Martinez, A. a., Agarwal, S., Zhang, Y., Sinha-Ray, S., Yarin, A.L., Greiner, A., Schröder, U., 2011. Electrospun and solution blown three-dimensional carbon fiber nonwovens for application as electrodes in microbial fuel cells. Energy & Environmental Science 4.

Cheng, C.-H., Hung, C.-H., Lee, K.-S., Liau, P.-Y., Liang, C.-M., Yang, L.-H., Lin, P.-J., Lin, C.-Y., 2008. Microbial community structure of a starch-feeding fermentative hydrogen production reactor operated under different incubation conditions. International Journal of Hydrogen Energy 33, 5242–5249.

Cheng, S., Kiely, P., Logan, B.E., 2011. Pre-acclimation of a wastewater inoculum to cellulose in an aqueous-cathode MEC improves power generation in air-cathode MFCs. Bioresource technology 102, 367–371.

Chiao, M., Lam, K.B., Lin, L., 2006. Micromachined microbial and photosynthetic fuel cells. Journal of Micromechanics and Microengineering 16, 2547–2553.

Choi, Y.-U., Kwon, Y.-K., Ye, B.-R., Hyun, J.-H., Heo, S.-J., Affan, A., Yoon, K.-T., Park, H.-S., Oh, C., Kang, D.-H., 2012. Draft genome sequence of *Marinobacterium stanieri* S30, a strain isolated from a coastal lagoon in Chuuk state in Micronesia. Journal of Bacteriology 194, 1260.

Cohen, B., 1931. The bacterial culture as an electrical half-cell. Journal of Bacteriology 21, 1–18.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms : A common cause of persistent infections. Science 284, 1318–1322.

Damien, A., 1992. Hydrogène par électrolyse de l'eau. Techniques de l'ingénieur 6, 1–9.

Das, D., Veziroglu, T.N., 2001. Hydrogen production by biological processes : a survey of literature. International Journal of Hydrogen Energy 26, 13–28.

Dumas, C., Basseguy, R., Bergel, A., 2008a. Electrochemical activity of *Geobacter sulfurreducens* biofilms on stainless steel anodes. Electrochimica Acta 53, 5235–5241.

Dumas, C., Basseguy, R., Bergel, A., 2008b. DSA to grow electrochemically active biofilms of *Geobacter sulfurreducens*. Electrochimica Acta 53, 3200–3209.

Erable, B., Roncato, M., Achouak, W., Bergel, A., 2009. Sampling Natural Biofilms: A New Route to Build Efficient Microbial Anodes. Environmental Science & Technology 43, 3194–3199.

Fang, H., Liu, H., 2002. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresource Technology 82, 87–93.

Fang, H., Liu, H., Zhang, T., 2005. Phototrophic hydrogen production from acetate and butyrate in wastewater. International Journal of Hydrogen Energy 30, 785–793.

Feijoo, G., Soto, M., Méndez, R., Lema, J.M., 1995. Sodium inhibition in the anaerobic digestion process: Antagonism and adaptation phenomena. Enzyme and Microbial Technology 17, 180–188.

Flemming, H.-C., 2002. Biofouling in water systems--cases, causes and countermeasures. Applied Microbiology and Biotechnology 59, 629–640.

Fredrickson, J.K., Romine, M.F., Beliaev, A.S., Jennifer, M., Osterman, L., Pinchuk, G., Reed, J.L., Rodionov, D.A., Jorge, L.M., 2008. Towards environmental systems biology of Shewanella. Nature Reviews Microbiology 6, 592–603.

Freguia, S., Masuda, M., Tsujimura, S., Kano, K., 2009. *Lactococcus lactis* catalyses electricity generation at microbial fuel cell anodes via excretion of a soluble quinone. Bioelectrochemistry 76, 14–18.

Freguia, S., Rabaey, K., Yuan, Z., Keller, J., 2007. Electron and carbon balances in microbial fuel cells reveal temporary bacterial storage behavior during electricity generation. Environmental Science & Technology 41, 2915–2921.

Freguia, S., Rabaey, K., Yuan, Z., Keller, J., 2008. Syntrophic processes drive the conversion of glucose in microbial fuel cell anodes. Environmental Science & Technology 42, 7937–7943.

Freguia, S., Teh, E.H., Boon, N., Leung, K.M., Keller, J., Rabaey, K., 2010. Microbial fuel cells operating on mixed fatty acids. Bioresource Technology 101, 1233–1238.

Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Forestier, N., Gillet, F., Aragno, M., Rossi, P., 2007. Minireview statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environmental Microbiology 4, 634–643.

Gavin, R., Merino, S., Altarriba, M., Canals, R., Shaw, J.G., TomÃ¡s, J.M., 2003. Lateral flagella are required for increased cell adherence, invasion and biofilm formation by *Aeromonas* spp. FEMS Microbiology Letters 224, 77–83.

Gebauer, R., 2004. Mesophilic anaerobic treatment of sludge from saline fish farm effluents with biogas production. Bioresource Technology 93, 155–167.

Girbal, L., Croux, C., Vasconcelos, I., Soucaille, P., 1995. Regulation of Metabolic Shifts in *Clostridium acetobutylicum* Atcc-824. FEMS Microbiology Reviews 17, 287–297.

Godon, J.-J., Zumstein, E., Dabert, P., Habouzit, F., 1997. Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis . Applied and Environmental Microbiology 63, 2802–2813.

Gorby, Y. a, Yanina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A., Beveridge, T.J., Chang, I.S., Kim, B.H., Kim, K.S., Culley, D.E., Reed, S.B., Romine, M.F., Saffarini, D. a, Hill, E. a, Shi, L., Elias, D. a, Kennedy, D.W., Pinchuk, G., Watanabe, K., Ishii, S., Logan, B., Nealson, K.H., Fredrickson, J.K., 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proceedings of the National Academy of Sciences of the United States of America 103, 11358–11363.

Greene, A.C., Patel, B.K.C., Yacob, S., 2009. *Geoalkalibacter subterraneus* sp. nov., an anaerobic Fe(III)- and Mn(IV)-reducing bacterium from a petroleum reservoir, and emended descriptions of the family *Desulfuromonadaceae* and the genus *Geoalkalibacter*. International Journal of Systematic and Evolutionary Microbiology 59, 781–785.

Guerrero, L., Omil, F., Méndez, R., Lema, J.M., 1997. Treatment of saline wastewaters from fish meal factories in an anaerobic filter under extreme ammonia concentrations. Bioresource Technology 61, 69–78.

Guo, X.M., Trably, E., Latrille, E., Carrère, H., Steyer, J.-P., 2010. Hydrogen production from agricultural waste by dark fermentation: A review. International Journal of Hydrogen Energy 35, 10660–10673.

Gómez, X., Fernández, C., Fierro, J., Sánchez, M.E., Escapa, A., Morán, A., 2011. Hydrogen production: two stage processes for waste degradation. Bioresource technology 102, 8621–8627.

Ha, P.T., Tae, B., Chang, I.S., 2008. Performance and bacterial consortium of microbial fuel cell fed with formate. Energy & Fuels 658–662.

Hajjaji, N., Pons, M., Renaudin, V., Houas, A., 2013. Comparative life cycle assessment of eight alternatives for hydrogen production from renewable and fossil feedstock. Journal of Cleaner Production 44, 177–189.

Hallenbeck, P.C., 2009. Fermentative hydrogen production: Principles, progress, and prognosis. International Journal of Hydrogen Energy 34, 7379–7389.

Hallenbeck, P.C., Benemann, J.R., 2002. Biological hydrogen production ; fundamentals and limiting processes. International Journal of Hydrogen Energy 27, 1185–1193.

Hallenbeck, P.C., Ghosh, D., 2009. Advances in fermentative biohydrogen production: the way forward? Trends in Biotechnology 27, 287–297.

Hamelers, H.V.M., Ter Heijne, A., Sleutels, T.H.J. a, Jeremiasse, A.W., Strik, D.P.B.T.B., Buisman, C.J.N., 2010. New applications and performance of bioelectrochemical systems. Applied Microbiology and Biotechnology 85, 1673–1685.

Harnisch, F., Koch, C., Patil, S. a., Hübschmann, T., Müller, S., Schröder, U., 2011. Revealing the electrochemically driven selection in natural community derived microbial biofilms using flow-cytometry. Energy & Environmental Science 4, 1265.

Harnisch, F., Rabaey, K., 2012. The diversity of techniques to study electrochemically active biofilms highlights the need for standardization. ChemSusChem 5, 1027–1038.

Hawkes, F., Hussy, I., Kyazze, G., Dinsdale, R., Hawkes, D., 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. International Journal of Hydrogen Energy 32, 172–184.

He, G., Gu, Y., He, S., Schröder, U., Chen, S., Hou, H., 2011. Effect of fiber diameter on the behavior of biofilm and anodic performance of fiber electrodes in microbial fuel cells. Bioresource technology 102, 10763–10766.

He, Z., Kan, J., Mansfeld, F., Angenent, L.T., Nealson, K.H., 2009. Self-sustained phototrophic microbial fuel cells based on the synergistic cooperation between photosynthetic microorganisms and heterotrophic bacteria. Environmental science & technology 43, 1648–1654.

Holmes, D., Nicoll, J., Bond, D., Lovley, D., 2004a. Potential role of a novel psychrotolerant member of the family Geobacteraceae , *Geopsychrobacter electrodiphilus* gen . nov ., sp . nov ., in electricity production by a marine sediment fuel cell. Applied Environmental Microbiology 70, 6023–6030.

Holmes, D.E., Bond, D.R., Lovley, D.R., 2004. Electron transfer by Desulfobulbus propionicus to Fe (III) and graphite electrodes 70, 1234–1237.

Holmes, D.E., Bond, D.R., O'Neil, R. a, Reimers, C.E., Tender, L.R., Lovley, D.R., 2004. Microbial communities associated with electrodes harvesting electricity from a variety of aquatic sediments. Microbial Ecology 48, 178–190.

Holmes, D.E., Finneran, K.T., Neil, R.A.O., Lovley, D.R., 2002. Enrichment of Members of the Family Geobacteraceae Associated with Stimulation of Dissimilatory Metal Reduction in Uranium-Contaminated Aquifer Sediments. Applied and Environmental Microbiology 68, 2300–2306.

Holmes, D.E., Nicoll, J.S., Bond, D.R., Lovley, D.R., 2004b. Potential role of a novel psychrotolerant member of the family Geobacteraceae, *Geopsychrobacter electrodiphilus* gen. nov., sp nov., in electricity production by a marine sediment fuel cell. Applied and Environmental Microbiology 70, 6023–6030.

Huang, L., Logan, B.E., 2008. Electricity production from xylose in fed-batch and continuous-flow microbial fuel cells. Applied Microbiology and Biotechnology 80, 655–664.

Ishii, S., Kosaka, T., Hori, K., Hotta, Y., 2005. Coaggregation facilitates interspecies hydrogen transfer between *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus*. Applied and Environmental Microbiology 71, 7838–7845.

Ishii, S., Shimoyama, T., Hotta, Y., Watanabe, K., 2008. Characterization of a filamentous biofilm community established in a cellulose-fed microbial fuel cell. BMC microbiology 12, 1–12.

Jang, J.K., Chang, I.S., Hwang, H.Y., Choo, Y.F., Lee, J., Cho, K.S., Kim, B.H., Nealson, K.H., 2010. Electricity generation coupled to oxidation of propionate in a microbial fuel cell. Biotechnology Letters 32, 79–85.

Jo, J.H., Lee, D.S., Park, D., Park, J.M., 2008. Biological hydrogen production by immobilized cells of *Clostridium tyrobutyricum* JM1 isolated from a food waste treatment process. Bioresource Technology 99, 6666–6672.

Jouenne, T., 2008. Biofilms bactériens. Techniques de l'ingénieur.

Jung, S., Regan, J.M., 2007. Comparison of anode bacterial communities and performance in microbial fuel cells with different electron donors. Applied Microbiology and Biotechnology 77, 393–402.

Katsikogianni, M., Missirlis, Y.F., 2004. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. European Cells & Materials 8, 37–57.

Ketep, S.F., Bergel, A., Bertrand, M., Achouak, W., Fourest, E., 2013. Lowering the applied potential during successive scratching/re-inoculation improves the performance of microbial anodes for microbial fuel cells. Bioresource Technology 127, 448–455.

Kiely, P.D., Call, D.F., Yates, M.D., Regan, J.M., Logan, B.E., 2010. Anodic biofilms in microbial fuel cells harbor low numbers of higher-power-producing bacteria than abundant genera. Applied Microbiology and Biotechnology 88, 371–380.

Kiely, P.D., Cusick, R., Call, D.F., Selembo, P. a, Regan, J.M., Logan, B.E., 2011. Anode microbial communities produced by changing from microbial fuel cell to microbial electrolysis cell operation using two different wastewaters. Bioresource Technology 102, 388–394.

Kiely, P.D., Rader, G., Regan, J.M., Logan, B.E., 2011. Long-term cathode performance and the microbial communities that develop in microbial fuel cells fed different fermentation endproducts. Bioresource Technology 102, 361–336.

Kiely, P.D., Regan, J.M., Logan, B.E., 2011. The electric picnic: synergistic requirements for exoelectrogenic microbial communities. Current Opinion in Biotechnology 22, 378–385.

Kim, B.-H., Kim, H.-J., Hyun, M.-S., Park, D.-H., 1999. Direct electrode reaction of Fe(III)-reducing bacterium, *Shewanella putrefaciens*. Microbiology and Biotechnology 9, 127–131.

Kim, B.H., Park, H.S., Kim, H.J., Kim, G.T., Chang, I.S., Lee, J., Phung, N.T., 2004. Enrichment of microbial community generating electricity using a fuel-cell-type electrochemical cell. Applied Microbiology and Biotechnology 63, 672–681.

Kim, D., Han, S., Kim, S., Shin, H., 2006. Effect of gas sparging on continuous fermentative hydrogen production. International Journal of Hydrogen Energy 31, 2158–2169.

Kim, G.T., Hyun, M.S., Chang, I.S., Kim, H.J., Park, H.S., Kim, B.H., Kim, S.D., Wimpenny, J.W.T., Weightman, a J., 2005. Dissimilatory Fe(III) reduction by an electrochemically active lactic acid bacterium phylogenetically related to *Enterococcus gallinarum* isolated from submerged soil. Journal of Applied Microbiology 99, 978–987.

Kim, H.-J., Hyun, M.-S., Chang, I.-S., Kim, B.-H., 1999. A microbial fuel cell type lactate biosensor using a metal-reducing bacterium, *Shewanella putrefaciens*. Microbiology and Biotechnology 9, 365–367.

Kim, J., Min, B., Logan, B., 2005. Evaluation of procedures to acclimate a microbial fuel cell for electricity production. Applied MIcrobiology and Biotechnology 68, 23–30.

Kim, J.R., Jung, S.H., Regan, J.M., Logan, B.E., 2007. Electricity generation and microbial community analysis of alcohol powered microbial fuel cells Bioresource Technology, 98, 2568–2577.

Kivisto, A., Santala, V., Karp, M., 2010. Hydrogen production from glycerol using halophilic fermentative bacteria. Bioresource Technology 101, 8671–8677.

Kolb, S., Seeliger, S., Springer, N., Ludwig, W., Schink, B., 1998. The fermenting bacterium *Malonomonas rubra* is phylogenetically related to sulfur-reducing bacteria and contains a c-type cytochrome similar to those of sulfur and sulfate reducers. Systematic and Applied Microbiology 21, 340–345.

Kumar, N., Ghosh, A., Das, D., 2001. Redirection of biochemical pathways for the enhancement of H2 production by *Enterobacter cloacae*. Biotechnology Letters 23, 537–541.

Lalaurette, E., Thammannagowda, S., Mohagheghi, A., Maness, P., Logan, B., 2009. Hydrogen production from cellulose in a two-stage process combining fermentation and electrohydrogenesis. International Journal of Hydrogen Energy 34, 6201–6210.

Latrille, E., Trably, E., Larroche, C., 2011. Production de biohydrogène : voie fermentaire sombre. Techniques de l'ingénieur.

Lay, J., Fan, K., Chang, J.-, Ku, C., 2003. Influence of chemical nature of organic wastes on their conversion to hydrogen by heat-shock digested sludge. International Journal of Hydrogen Energy 28, 1361–1367.

Lay, J.J., 2000. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnology and bioengineering 68, 269–278.

Lee, H., Torres, C., Parameswaran, P., Rittmann, B., 2009. Fate of H2 in an upflow single-chamber microbial electrolysis cell using a metal-catalyst-free cathode. Environmental Science & Technology 43, 7971–7976.

Lee, M., Kim, T., Min, B., Hwang, S., 2012. Sodium (Na+) concentration effects on metabolic pathway and estimation of ATP use in dark fermentation hydrogen production through stoichiometric analysis. Journal of Environmental Management 108, 22–26.

Lefebvre, O., Moletta, R., 2006. Treatment of organic pollution in industrial saline wastewater: a literature review. Water research 40, 3671–3682.

Lefebvre, O., Quentin, S., Torrijos, M., Godon, J.J., Delgenès, J.P., Moletta, R., 2007. Impact of increasing NaCl concentrations on the performance and community composition of two anaerobic reactors. Applied Microbiology and Biotechnology 75, 61–69.

Lefebvre, O., Tan, Z., Kharkwal, S., Ng, H.Y., 2012. Effect of increasing anodic NaCl concentration on microbial fuel cell performance. Bioresource Technology 112, 336–340.

Lefebvre, O., Uzabiaga, A., Chang, I.S., 2010. Microbial fuel cells for energy self-sufficient domestic wastewater treatment — a review and discussion from energetic consideration. Applied Microbiology and Biotechnology 89, 259–270.

Lefebvre, O., Uzabiaga, A., Chang, I.S., Kim, B.-H., Ng, H.Y., 2011. Microbial fuel cells for energy self-sufficient domestic wastewater treatment-a review and discussion from energetic consideration. Applied Microbiology and Biotechnology 89, 259–270.

Lefebvre, O., Vasudevan, N., Torrijos, M., Thanasekaran, K., Moletta, R., 2006. Anaerobic digestion of tannery soak liquor with an aerobic post-treatment. Water Research 40, 1492–500.

Lenin Babu, M., Venkata Subhash, G., Sarma, P.N., Venkata Mohan, S., 2013. Bio-electrolytic conversion of acidogenic effluents to biohydrogen: An integration strategy for higher substrate conversion and product recovery. Bioresource Technology 133, 322–331.

Levin, D., Pitt, L., Love, M., 2004. Biohydrogen production: prospects and limitations to practical application. International Journal of Hydrogen Energy 29, 173–185.

Li, A., Chu, Y., Wang, X., Ren, L., Yu, J., Liu, X., Yan, J., Zhang, L., Wu, S., Li, S., 2013. A pyrosequencing-based metagenomic study of methane-producing microbial community in solid-state biogas reactor. Biotechnology for Biofuels 6, 1–17.

Li, C., Fang, H.H.P., Journals, P.S., 2007. Fermentative Hydrogen Production From Wastewater and Solid Wastes by Mixed Cultures Critical Reviews in Environmental Science and Technology 37, 1–39.

Lin, C., Chang, C., Hung, C., 2008. Fermentative hydrogen production from starch using natural mixed cultures. International Journal of Hydrogen Energy 33, 2445–2453.

Liu, D., Min, B., Angelidaki, I., 2008. Biohydrogen production from household solid waste (HSW) at extreme-thermophilic temperature (70°C) – Influence of pH and acetate concentration. International Journal of Hydrogen Energy 33, 6985–6992.

Liu, H., Cheng, S., Logan, B.E., 2005a. Production of electricity from acetate or butyrate using a single-chamber microbial fuel cell. Environmental Science & Technology 39, 658–662.

Liu, H., Cheng, S., Logan, B.E., 2005b. Power generation in fed-batch microbial fuel cells as a function of ionic strength, temperature, and reactor configuration. Environmental Science & Technology 39, 5488–5493.

Liu, H., Grot, S., Logan, B.E., 2005. Electrochemically assisted microbial production of hydrogen from acetate. Environmental Science & Technology 39, 4317–4320.

Liu, H., Hu, H., Chignell, J., YanZhen, F., Fan, Y., 2010. Microbial electrolysis: novel technology for hydrogen production from biomass. Biofuels 1, 129–142.

Liu, H., Wang, G., 2012. Hydrogen production of a salt tolerant strain *Bacillus* sp. B2 from marine intertidal sludge. World Journal of Microbiology & Biotechnology 28, 31–7.

Liu, W., Huang, S., Zhou, A., Zhou, G., Ren, N., Wang, A., Zhuang, G., 2012. Hydrogen generation in microbial electrolysis cell feeding with fermentation liquid of waste activated sludge. International Journal of Hydrogen Energy 37, 13859–13864.

Liu, W., Wang, A., Sun, D., Ren, N., Zhang, Y., Zhou, J., 2012. Characterization of microbial communities during anode biofilm reformation in a two-chambered microbial electrolysis cell (MEC). Journal of Biotechnology 157, 628–632.

Logan, B., 2009. Exoelectrogenic bacteria that power microbial fuel cells. Nature Reviews Microbiology 7, 375–381.

Logan, B.E., 2010. Scaling up microbial fuel cells and other bioelectrochemical systems. Applied Microbiology and Biotechnology 85, 1665–1671.

Logan, B.E., 2012. Essential data and techniques for conducting microbial fuel cell and other types of bioelectrochemical system experiments. ChemSusChem 5, 988–994.

Logan, B.E., Call, D., Cheng, S., Hamelers, H.V.M.H., Sleutels, T.H.J. a. T., Jeremiasse, A.A.W., Rozendal, R. a. R., 2008. Microbial electrolysis cells for high yield hydrogen gas production from organic matter. Environmental Science & Technology 42, 8630–8640.

Logan, B.E., Regan, J.M., 2006. Electricity-producing bacterial communities in microbial fuel cells. Trends in Microbiology 14, 512–518.

Lovley, D., 2006a. Bug juice: harvesting electricity with microorganisms. Nature Reviews Microbiology 4, 497–508.

Lovley, D., 2008. The microbe electric: conversion of organic matter to electricity. Current Opinion in Biotechnology 19, 564–571.

Lovley, D.D.R., 2006b. Bug juice: harvesting electricity with microorganisms. Nature Reviews Microbiology 4, 497–508.

Lovley, D.R., 1993. Dissimilatory metal reduction. Annual Review of Microbiology 47, 263–290.

Lovley, D.R., 2011. Live wires: direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. Energy & Environmental Science 4, 4896.

Lovley, D.R., 2012. Electromicrobiology. Annual Review of Microbiology 66, 391–409.

Lovley, D.R., Phillips, E.J., 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Applied and Environmental Microbiology 51, 683–689.

Lower, B.H., Hochella, M.F., Lower, S.K., 2005. Putative mineral-specific proteins synthesizes by metal reducing bacterium. American Journal of Science 305, 687–710.

Lu, L., Ren, N., Xing, D., Logan, B., 2009. Hydrogen production with effluent from an ethanol-H2-coproducing fermentation reactor using a single-chamber microbial electrolysis cell. Biosensors and Bioelectronics 24, 3055–3060.

Lu, L., Xing, D., Ren, N., 2012. Pyrosequencing reveals highly diverse microbial communities in microbial electrolysis cells involved in enhanced H2 production from waste activated sludge. Water research 46, 2425–34.

Malki, M., Gonza, E., Go, F., Amils, R., 2006. The isolation and study of acidophilic microorganisms. Methods in Microbiology 35, 35020–35023.

Mariani, C., Oulahal, N., Chamba, J.-F., Dubois-Brissonnet, F., Notz, E., Briandet, R., 2011. Inhibition of Listeria monocytogenes by resident biofilms present on wooden shelves used for cheese ripening. Food Control 22, 1357–1362.

Marshall, C., May, H., 2009. Electrochemical evidence of direct electrode reduction by a thermophilic Gram-positive bacterium, *Thermincola ferriacetica*. Energy & Environmental Science 2, 699–705.

Marsili, E., Rollefson, J.B., Baron, D.B., Hozalski, R.M., Bond, D.R., 2008. Microbial biofilm voltammetry: direct electrochemical characterization of catalytic electrode-attached biofilms. Applied and Environmental Microbiology 74, 7329–7337.

Marsili, E., Sun, J., Bond, D.R., 2010. Voltammetry and growth physiology of *Geobacter sulfurreducens* biofilms as a function of growth stage and imposed electrode potential. Electroanalysis 22, 865–874.

Maukonen, J., Mättö, J., Wirtanen, G., Raaska, L., Mattila-Sandholm, T., Saarela, M., 2003. Methodologies for the characterization of microbes in industrial environments: a review. Journal of Industrial Microbiology & Biotechnology 30, 327–356.

Miceli, J.F., Parameswaran, P., Kang, D.-W., Krajmalnik-Brown, R., Torres, C.I., 2012. Enrichment and analysis of anode-respiring bacteria from diverse anaerobic inocula. Environmental Science & Technology 46, 10349–10355.

Michelland, R.J., Dejean, S., Combes, S., Fortun-Lamothe, L., Cauquil, L., 2009. StatFingerprints: a friendly graphical interface program for processing and analysis of microbial fingerprint profiles. Molecular Ecology Ressources 9, 1359–1363.

Millet, P., 2008. Le réacteur électrochimique. Techniques de l'ingénieur.

Milliken, C., May, H., 2007. Sustained generation of electricity by the spore-forming, Gram-positive, *Desulfitobacterium hafniense* strain DCB2. Applied Microbial and Cell Physiology 73, 1180–1189.

Min, B., Logan, B.E., 2004. Continuous electricity generation from domestic wastewater and organic substrates in a flat plate microbial fuel cell. Environmental Science & Technology 38, 5809–5814.

Monlau, F., Barakat, A., Steyer, J.P., Carrere, H., 2012. Comparison of seven types of thermo-chemical pretreatment on the structural features and anaerobic digestion of sunflower stalks. Bioresource Technology 120, 241–247.

Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P., Carrère, H., 2013. Lignocellulosic materials into biohydrogen and biomethane: impact of structural features and pretreatment. Critical Reviews in Environmental Science and Technology 43, 260–322.

Mosquera-Corral, A., Sanchez, M., 2001. Simultaneous methanogenesis and denitrification of pretreated effluents from a fish canning industry. Water Research 35, 411–418.

Nealson, K.H., Saffarini, D., 1994. Iron and manganese in anaerobic respiration: environmental significance physiology and regulation. Annual Review of Microbiology 48, 311–343.

Nercessian, O., Parot, S., Délia, M.-L., Bergel, A., Achouak, W., 2012. Harvesting Electricity with *Geobacter bremensis* isolated from compost. PLoS One 7.

Nevin, K., Kim, B., Glaven, R., Johnson, J., Woodard, T., Methe, B., DiDonato, R., Covalla, S., Franks, A., Liu, A., Lovley, D., 2009. Anode biofilm transcriptomics reveals outer surface components essential for high density current production in *Geobacter sulfurreducens*. Fuel Cells. PloS one 4.

Nevin, K.P., Richter, H., Covalla, S.F., Johnson, J.P., Woodard, T.L., Orloff, a L., Jia, H., Zhang, M., Lovley, D.R., 2008. Power output and columbic efficiencies from biofilms of *Geobacter sulfurreducens* comparable to mixed community microbial fuel cells. Environmental Microbiology 10, 2505–2514.

Niessen, J., Schroder, U., Scholz, F., 2004. Exploiting complex carbohydrates for microbial electricity generation ? A bacterial fuel cell operating on starch. Electrochemistry Communications 6, 955–958.

Noike, T., Takabatake, H., Mizuno, O., Ohba, M., 2002. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. International Journal of Hydrogen Energy 27, 1367–1371.

Ntaikou, I., Antonopoulou, G., Lyberatos, G., 2010. Biohydrogen production from biomass and wastes via dark fermentation: A Review. Waste and Biomass Valorization 1, 21–39.

Oh, Y.-K., Park, M.S., Seol, E.-H., Lee, S.-J., Park, S., 2003. Isolation of hydrogen-producing bacteria from granular sludge of an upflow anaerobic sludge blanket reactor. Biotechnology and Bioprocess Engineering 8, 54–57.

Ohkawara, T., Hirose, J.U.N., Hayashi, S.A.T., 1995. of Hydrogen production by aciduric *Enterobacter aerogenes* Strain HO-39. Journal of Fermentation and Bioengineering 80, 571–574.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2010. Vegan : community ecology package.

Oren, A., 2001. The bioenergetic basis for the decrease in metabolic diversity at increasing salt concentrations: implications for the functioning of salt lake ecosystems. Hydrobiologia 466, 61–72.

O'Toole, G. a, Kolter, R., 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Molecular microbiology 30, 295–304.

Pant, D., Singh, A., Van Bogaert, G., Irving Olsen, S., Singh Nigam, P., Diels, L., Vanbroekhoven, K., 2012. Bioelectrochemical systems (BES) for sustainable energy production and product recovery from organic wastes and industrial wastewaters. RSC Advances 2, 1248–1263.

Parameswaran, P., Bry, T., Popat, S., Lusk, B.G., Rittmann, B.E., Torres, C.I., 2013. Kinetic, electrochemical, and microscopic characterization of the thermophilic, anode-respiring bacterium *Thermincola ferriacetica*. Environmental Science & Technology.

Parameswaran, P., Torres, C.I., Lee, H.-S., Krajmalnik-Brown, R., Rittmann, B.E., 2009. Syntrophic interactions among anode respiring bacteria (ARB) and non-ARB in a biofilm anode: Electron balances. Biotechnology and Bioengineering 103, 513–523.

Parameswaran, P., Zhang, H., Torres, C.I., Rittmann, B.E., Krajmalnik-Brown, R., 2010. Microbial community structure in a biofilm anode fed with a fermentable substrate: the significance of hydrogen scavengers. Biotechnology and Bioengineering 105, 69–78.

Park, H.S., Kim, B.H., Kim, H.S., Kim, H.J., Kim, G.T., Kim, M., Chang, I.S., Park, Y.K., Chang, H.I., 2001. A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to *Clostridium butyricum* isolated from a microbial fuel cell. Anaerobe 7, 297–306.

Patil, S. a., Hägerhäll, C., Gorton, L., 2012. Electron transfer mechanisms between microorganisms and electrodes in bioelectrochemical systems. Bioanalytical Reviews 4, 159–192.

Patil, S. a, Harnisch, F., Koch, C., Hübschmann, T., Fetzer, I., Carmona-Martínez, A. a, Müller, S., Schröder, U., 2011. Electroactive mixed culture derived biofilms in microbial bioelectrochemical systems: the role of pH on biofilm formation, performance and composition. Bioresource Technology 102, 9683–9690.

Pfennig, N., Biebl, H., 1976. *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Archives of Microbiology 110, 3–12.

Pham, C.A.C., Jung, S.J.S., Phung, N.T.N., Lee, J., Chang, I.S.I., Kim, B.B.H., Yi, H., Chun, J., 2003. A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to *Aeromonas hydrophila*, isolated from a microbial fuel cell. FEMS Microbiology Letters 223, 129–134.

Pierra, M., Trably, E., Bonnafous, A., Godon, J.-J., Bernet, N., 2012. High selection and identification of electricigen bacteria from graphite anode biofilm under a controlled potential: *Geoalkalibacter subterraneus* as a new electricigen., in: Communications in Agricultural and Applied Biological Sciences. p. 77.

Pierra, M., Trably, E., Godon, J.-J., Bernet, N., 2013. Fermentative hydrogen production under moderate halophilic conditions. International Journal of Hydrogen Energy. DOI: 10.1016/j.ijhydene.2013.08.035

Puig, S., Serra, M., Coma, M., Cabré, M., Balaguer, M.D., Colprim, J., 2010. Effect of pH on nutrient dynamics and electricity production using microbial fuel cells. Bioresource Technology 101, 9594–9599.

Qu, Y., Feng, Y., Wang, X., Logan, B.E., 2012. Using a co-culture to enable current production by *Geobacter sulfurreducens*. Applied and Environmental Microbiology 78, 3484–3487.

Quéméneur, M., Hamelin, J., Barakat, A., Steyer, J.-P., Carrère, H., Trably, E., 2012. Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures. International Journal of Hydrogen Energy 37, 3150–3159.

Quéméneur, M., Hamelin, J., Benomar, S., Guidici-Orticoni, M.-T., Latrille, E., Steyer, J.-P., Trably, E., 2011. Changes in hydrogenase genetic diversity and proteomic patterns in mixed-

culture dark fermentation of mono-, di- and tri-saccharides. International Journal of Hydrogen Energy 36, 11654– 11665.

Quéméneur, M., Hamelin, J., Latrille, E., Steyer, J.-P., Trably, E., 2010. Development and application of a functional CE-SSCP fingerprinting method based on [Fe–Fe]-hydrogenase genes for monitoring hydrogen-producing *Clostridium* in mixed cultures. International Journal of Hydrogen Energy 35, 13158–13167.

Quéméneur, M., Hamelin, J., Latrille, E., Steyer, J.-P., Trably, E., Benomar, S., Guidici-Orticoni, M.-T., 2011. Functional versus phylogenetic fingerprint analyses for monitoring hydrogen-producing bacterial populations in dark fermentation cultures. International Journal of Hydrogen Energy 36, 3870–3879.

R. Develpment Core Team, 2010. R: a language and environement for statistical computing.

Rabaey, K., Angenent, L.T., Schroder, U., Keller, J., 2010. Electrochemical techniques for the analysis of bioelectrochemical systems, in: Bioelectrochemical Systems: From Extracellular Electron Transfer to Biotechnological Application. pp. 135–184.

Rabaey, K., Boon, N., Siciliano, S., Verhaege, M., Verstraete, W., 2004. Biofuel cells select for microbial consortia that self-mediate electron transfer. Applied and Environmental Microbiology 70, 5373–5382.

Rabaey, K., Verstraete, W., 2005. Microbial fuel cells: novel biotechnology for energy generation. Trends in Biotechnology 23, 291–298.

Rafrafi, Y., 2010. Impact des facteurs biotiques sur le réseau métabolique des écosystèmes producteurs d'hydrogène par voie fermentaire en culture mixte. Thèse de l'Université de Montpellier 2.

Rafrafi, Y., Trably, E., Hamelin, J., Latrille, E., Meynial-Salles, I., Benomar, S., Giudici-Orticoni, M.-T., Steyer, J.-P., 2013. Sub-dominant bacteria as keystone species in microbial communities producing bio-hydrogen. International Journal of Hydrogen Energy 38, 4975–4985.

Raghavulu, S.V., Sarma, P.N., Mohan, S.V., 2011. Comparative bioelectrochemical analysis of *Pseudomonas aeruginosa* and *Escherichia coli* with anaerobic consortia as anodic biocatalyst for biofuel cell application. Journal of Applied Microbiology 110, 666–674.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., Lovley, D.R., 2005. Extracellular electron transfer via microbial nanowires. Nature 435, 1098–1101.

Reguera, G., Nevin, K.P., Nicoll, J.S., Covalla, S.F., Woodard, T.L., Lovley, D.R., 2006. Biofilm and nanowire production leads to increased current in *Geobacter sulfurreducens* fuel cells. Applied and Environmental Microbiology 72, 7345–7348.

Rezaei, F., Xing, D., Wagner, R., Regan, J.M., Richard, T.L., Logan, B.E., 2009. Simultaneous cellulose degradation and electricity production by *Enterobacter cloacae* in a microbial fuel cell. Applied and Environmental Microbiology 75, 3673–3678.

Richter, H., Lanthier, M., Nevin, K.P., Lovley, D.R., 2007. Lack of electricity production by *Pelobacter carbinolicus* indicates that the capacity for Fe(III) oxide reduction does not necessarily

confer electron transfer ability to fuel cell anodes. Applied and Environmental Microbiology 73, 5347–5353.

Rittmann, B.E., 2008. Opportunities for renewable bioenergy using microorganisms. Biotechnology and Bioengineering 100, 203–212.

Roden, E.E., Lovley, D.R., 1993. Dissimilatory Fe(III) reduction by the marine microorganism *Desulfuromonas acetoxidans*. Applied and Environmental Microbiology 59, 734–742.

Rodríguez, J., Kleerebezem, R., Lema, J.M., van Loosdrecht, M.C.M., 2006. Modeling product formation in anaerobic mixed culture fermentations. Biotechnology and Bioengineering 93, 592–606.

Roling, W., Prasetyo, A., Stouthamer, A., Van Verseveld, 1999. Note: Physiological aspects of the growth of the lactic acid bacterium *Tetragenococcus halophila* during Indonesian soy sauce (kecap) production. Journal of Applied Microbiology 06, 348–352.

Rosenbaum, M., He, Z., Angenent, L.T., 2010. Light energy to bioelectricity: photosynthetic microbial fuel cells. Current Opinion in Biotechnology 21, 259–264.

Rousseau, R., Dominguez-Benetton, X., Délia, M.-L., Bergel, A., 2013. Microbial bioanodes with high salinity tolerance for microbial fuel cells and microbial electrolysis cells. Electrochemistry Communications 33, 1–4.

Rozendal, R., Hamelers, H.V.M.H., Molenkmp, R., Buisman, J.N., Rozendal, A., Molenkamp, R.J., 2007. Performance of single chamber biocatalyzed electrolysis with different types of ion exchange membranes. Water Research 41, 1984–1994.

Rozendal, R., Jeremiasse, A., Hamelers, H., Buisman, C., 2008. Hydrogen production with a microbial biocathode. Environmental Science & Technology 42, 629–634.

Rozendal, R.R.A., Hamelers, H.V.M.H., Euverink, G.J.W.G., Metz, S.S.J., Buisman, C.J.N.C., 2006. Principle and perspectives of hydrogen production through biocatalyzed electrolysis. International Journal of Hydrogen Energy 31, 1632–1640.

Saratale, G.D., Chen, S., Lo, Y., Saratale, R.G., Chang, J., 2008. Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation – a review 67, 962–979.

Schröder, U., Nießen, J., Scholz, F., Niessen, J., 2003. A generation of microbial fuel cells with current outputs boosted by more than one order of magnitude. Angewandte Chemie (International ed. in English) 42, 2880–2883.

Selembo, P. a. P., Perez, J.J.M., Lloyd, W. a., Logan, B.B.E., 2009. High hydrogen production from glycerol or glucose by electrohydrogenesis using microbial electrolysis cells. International Journal of Hydrogen Energy. 34, 5373–5381.

Sen, B., Hamelin, J., Bru-Adan, V., Godon, J.-J., Chandra, T.S., 2008. Structural divergence of bacterial communities from functionally similar laboratory-scale vermicomposts assessed by PCR-CE-SSCP. Journal of Applied Microbiology 105, 2123–2132.

Shukla, A.K., Suresh, P., Berchmans, S., Rajendran, A., 2004. Biological fuel cells and their applications. Current Science 87, 455–468.

Simankova, M., Chernych, N., Osipov, G., Zavarzin, G., 1993. *Halocella cellulolytica* gen. nov., sp. nov., a New Obligately Anaerobic, Halophilic, Cellulolytic bacterium. Systematic and Applied Microbiology 16, 385–389.

Simpson, E.H., 1949. Measurement of diversity. Nature 163, 688.

Sleutels, T.H.J. a, Darus, L., Hamelers, H.V.M., Buisman, C.J.N., 2011. Effect of operational parameters on Coulombic efficiency in bioelectrochemical systems. Bioresource Technology 102, 11172–11176.

Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W., 2002. Biofilms as complex differentiated communities. Annual Review of Microbiology 56, 187–209.

Temudo, M.F., Muyzer, G., Kleerebezem, R., van Loosdrecht, M.C.M., 2008. Diversity of microbial communities in open mixed culture fermentations: impact of the pH and carbon source. Applied Microbiology and Biotechnology 80, 1121–1130.

Tender, L.M., Reimers, C.E., Stecher, H. a, Holmes, D.E., Bond, D.R., Lowy, D. a, Pilobello, K., Fertig, S.J., Lovley, D.R., 2002. Harnessing microbially generated power on the seafloor. Nature Biotechnology 20, 821–825.

Tommasi, T., Ruggeri, B., Sanfilippo, S., 2012. Energy valorisation of residues of dark anaerobic production of Hydrogen. Journal of Cleaner Production 34, 91–97.

Torres, C.I., Krajmalnik-Brown, R., Parameswaran, P., Marcus, A.K., Wanger, G., Gorby, Y. a, Rittmann, B.E., 2009. Selecting anode-respiring bacteria based on anode potential: phylogenetic, electrochemical, and microscopic characterization. Environmental Science & Technology 43, 9519–9524.

Torres, C.I., Marcus, A.K., Rittmann, B.E., 2007. Kinetics of consumption of fermentation products by anode-respiring bacteria. Applied Microbiology and Biotechnology 77, 689–697.

Tuna, E., Kargi, F., Argun, H., 2009. Hydrogen gas production by electrohydrolysis of volatile fatty acid (VFA) containing dark fermentation effluent. International Journal of Hydrogen Energy 34, 262–269.

Turcot, J., Bisaillon, A., Hallenbeck, P., 2008. Hydrogen production by continuous cultures of *Escherchia coli* under different nutrient regimes. International Journal of Hydrogen Energy 33, 1465–1470.

Valdez-vazquez, I., Poggi-varaldo, M., 2009. Hydrogen production by fermentative consortia. Renewable and Sustainable Energy Reviews 13, 1000–1013.

Vallero, M.V.G., Hulshoff Pol, L.W., Lettinga, G., Lens, P.N.L., 2003. Effect of NaCl on thermophilic (55 degrees C) methanol degradation in sulfate reducing granular sludge reactors. Water Research 37, 2269–2280.

Van Niel, E.W.J., Claassen, P. a M., Stams, A.J.M., 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnology and Bioengineering 81, 255–262.

Vandieken, V., Mussmann, M., Niemann, H., Jørgensen, B.B., 2006. *Desulfuromonas svalbardensis* sp. nov. and *Desulfuromusa ferrireducens* sp. nov., psychrophilic, Fe(III)-reducing bacteria isolated from Arctic sediments, Svalbard. International Journal of Systematic and Evolutionary Microbiology 56, 1133–9.

Venkata Mohan, S., Prathima Devi, M., 2012. Fatty acid rich effluent from acidogenic biohydrogen reactor as substrate for lipid accumulation in heterotrophic microalgae with simultaneous treatment., Bioresource Technology, 123, 627-635

Wang, A., Sun, D., Cao, G., Wang, H., Ren, N., Wu, W.-M., Logan, B.E., 2011. Integrated hydrogen production process from cellulose by combining dark fermentation, microbial fuel cells, and a microbial electrolysis cell. Bioresource Technology 102, 4137–43.

Wang, A., Sun, D., Ren, N., Liu, C., Liu, W., Logan, B.E., Wu, W.-M., 2010. A rapid selection strategy for an anodophilic consortium for microbial fuel cells. Bioresource Technology 101, 5733–5735.

Wang, J., Wan, W., 2009a. Factors influencing fermentative hydrogen production: A review. International Journal of Hydrogen Energy 34, 799–811.

Wang, J., Wan, W., 2009b. Experimental design methods for fermentative hydrogen production: A review. International Journal of Hydrogen Energy 34, 235–244.

Wang, J., Wan, W., 2009c. Kinetic models for fermentative hydrogen production: A review. International Journal of Hydrogen Energy 34, 3313–3323.

Wang, X., Feng, Y., Wang, H., Qu, Y., Yu, Y., Ren, N., Li, N., Wang, E., Lee, H., Logan, B.E., 2009. Bioaugmentation for electricity generation from corn stover biomass using microbial fuel cells. Environmental Science & Technology 43, 6088–93.

Weber, K. a, Achenbach, L. a, Coates, J.D., 2006. Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. Nature Reviews Microbiology 4, 752–64.

Wei, J., Liang, P., Cao, X., Huang, X., 2010. A new insight into potential regulation on growth and power generation of *Geobacter sulfurreducens* in microbial fuel cells based on energy viewpoint. Environmental Science & Technology 44, 3187–91.

White, H.K., Reimers, C.E., Cordes, E.E., Dilly, G.F., Girguis, P.R., 2009. Quantitative population dynamics of microbial communities in plankton-fed microbial fuel cells. The ISME journal 3, 635–46.

Wéry, N., Bru-Adan, V., Minervini, C., Delgénes, J.-P., Garrelly, L., Godon, J.-J., 2008. Dynamics of *Legionella* spp. and bacterial populations during the proliferation of *L. pneumophila* in a cooling tower facility. Applied and Environmental Microbiology 74, 3030–3037.

Xia, X., Cao, X., Liang, P., Huang, X., Yang, S., Zhao, G., 2010. Electricity generation from glucose by a *Klebsiella sp.* in microbial fuel cells. Applied Microbiology and Biotechnology 87, 383–90.

Xiao, Y., Roberts, D.J., 2010. A review of anaerobic treatment of saline wastewater. Environmental Technology 31, 1025–1043.

Xing, D., Cheng, S., Logan, B.E., Regan, J.M., 2010. Isolation of the exoelectrogenic denitrifying bacterium *Comamonas denitrificans* based on dilution to extinction. Applied Microbiology and Biotechnology 85, 1575–87.

Xing, D., Cheng, S., Regan, J.M., Logan, B.E., 2009. Change in microbial communities in acetate- and glucose-fed microbial fuel cells in the presence of light. Biosensors & Bioelectronics 25, 105–111.

Xing, D., Zuo, Y., Cheng, S.S., Regan, J.J.M., Logan, B., 2008. Electricity generation by *Rhodopseudomonas palustris* DX-1. Environmental Science & Technology 42, 4146–4151.

Yang, Y., Xu, M., Guo, J., Sun, G., 2012. Bacterial extracellular electron transfer in bioelectrochemical systems. Process Biochemistry 47, 1707–1714.

Yoon, J.-H., 2003. *Psychrobacter jeotgali* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. International Journal of Systematic and Evolutionary Microbiology 53, 449–454.

Yoon, J.-H., Lee, C.-H., Kang, S.-J., Oh, T.-K., 2005. *Psychrobacter celer* sp. nov., isolated from sea water of the South Sea in Korea. International journal of systematic and evolutionary microbiology 55, 1885–1890.

Yoon, J.-H., Lee, C.-H., Yeo, S.-H., Oh, T.-K., 2005. *Psychrobacter aquimaris* sp. nov. and *Psychrobacter namhaensis* sp. nov., isolated from sea water of the South Sea in Korea. International Journal of Systematic and Evolutionary Microbiology 55, 1007–1013.

Yuan, W., Ágoston, R., Lee, D., Lee, S.-C., Yuk, H.-G., 2012. Influence of lactate and acetate salt adaptation on *Salmonella Typhimurium* acid and heat resistance. Food microbiology 30, 448–452.

Yuan, Y., Zhao, B., Zhou, S., Zhong, S., Zhuang, L., 2011. Electrocatalytic activity of anodic biofilm responses to pH changes in microbial fuel cells. Bioresource Technology 102, 6887–6891.

Zhang, C., Lv, F.-X., Xing, X.-H., 2011. Bioengineering of the *Enterobacter aerogenes* strain for biohydrogen production. Bioresource Technology 102, 8344–8349.

Zhang, L., Zhou, S., Zhuang, L., Li, W., Zhang, J., Lu, N., Deng, L., 2008. Microbial fuel cell based on *Klebsiella pneumoniae* biofilm. Electrochemistry Communications 10, 1641–1643.

Zhang, T., Cui, C., Chen, S., Ai, X., Yang, H., Shen, P., Peng, Z., 2006. A novel mediatorless microbial fuel cell based on direct biocatalysis of *Escherichia coli*. Chemical Communications 2257–2259.

Zhao, Y., Watanabe, K., Nakamura, R., Mori, S., Liu, H., Ishii, K., Hashimoto, K., 2010. Three-dimensional conductive nanowire networks for maximizing anode performance in microbial fuel cells. Chemistry (Weinheim an der Bergstrasse, Germany) 16, 4982–4985.

Zheng, X.J., Zheng, Y.M., Yu, H.Q., 2005. Influence of NaCl on hydrogen production from glucose by anaerobic cultures. Environmental Technology 26, 1073–1080.

Zobell, C.E., 1943. The effect of solid surfaces upon bacterial activity. Journal of Bacteriology 39–56.

Zuo, Y., Maness, P., Logan, B.E., 2006. Electricity production from steam-exploded corn stover biomass. Energy & Fuels 20, 1716–1721.

Zuo, Y., Xing, D., Regan, J., Logan, B., 2008. Isolation of the exoelectrogenic bacterium *Ochrobactrum anthropi* YZ-1 by using a U-tube microbial fuel cell. Applied and Environmental Microbiology 74, 3130–3137.

ANNEXES

ANNEXE 1

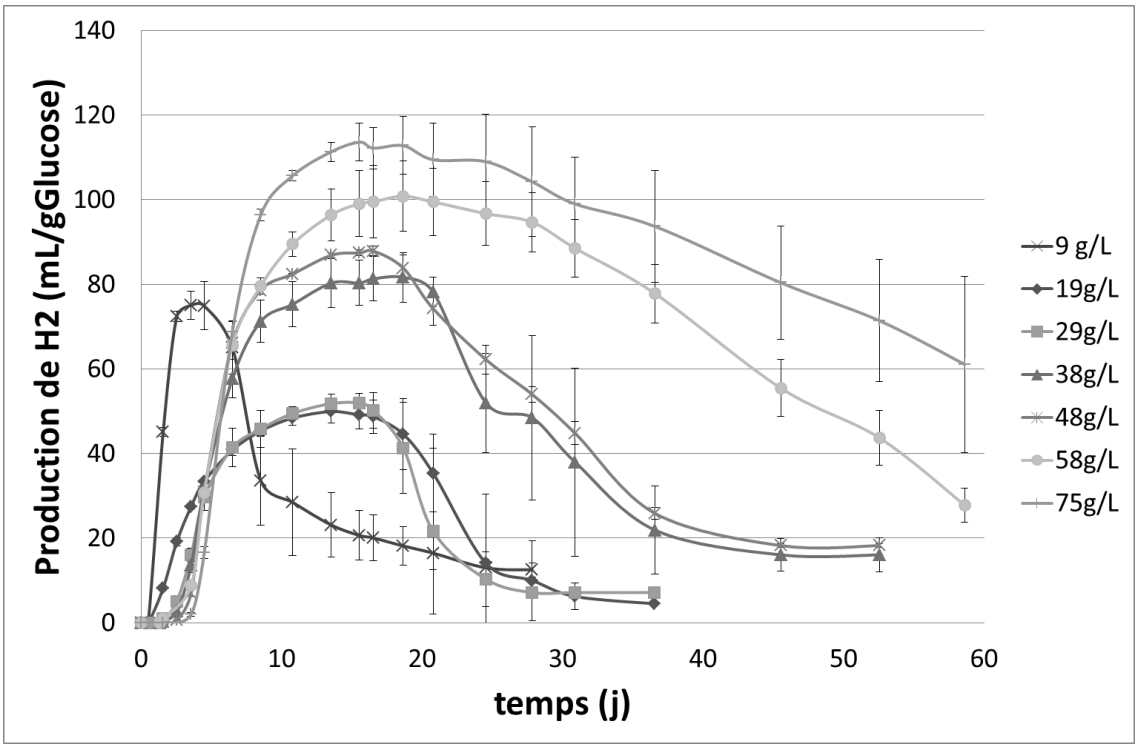


Figure Annexe-7 : Production d'hydrogène en $mL_{H2}/mol_{Glucose}$ en fonction du temps dans les essais à pH 8 pour les différentes salinités testées

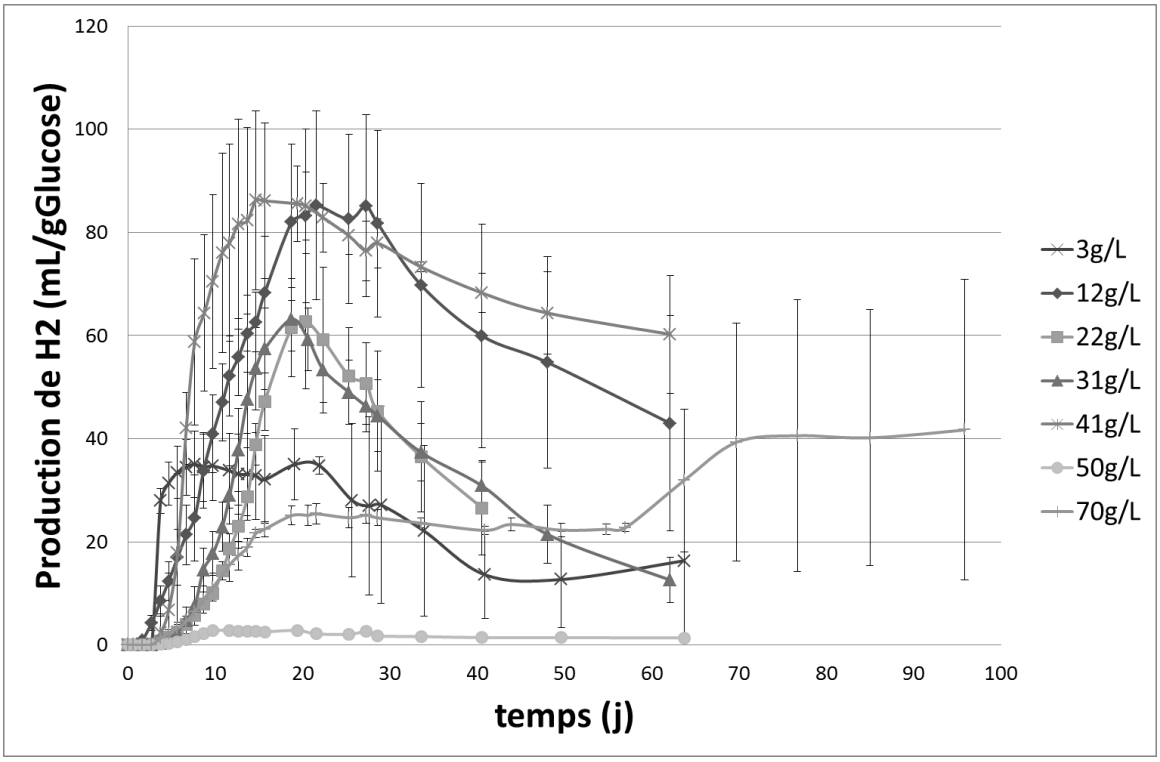


Figure Annexe-8 : Production d'hydrogène en $mL_{H2}/mol_{Glucose}$ en fonction du temps dans les essais à pH 6 pour les différentes salinités testées.

ANNEXE 2

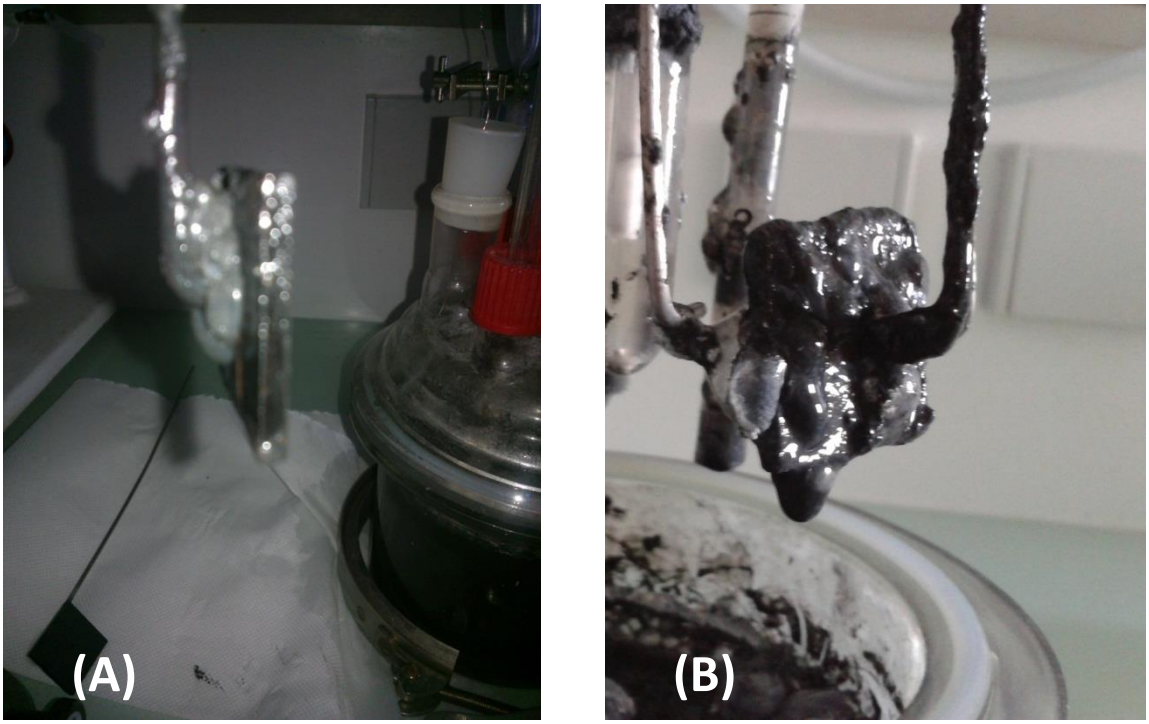


Figure Annexe-9 : Images des biofilms électroactifs lors de l'expérience réalisée sur la contamination d'espèces exogènes sur le biofilm.

(A) Biofilm RB8-1 prélevé en fin de premier cycle, juste avant l'ajout d'espèces exogènes dans le réacteur RB8, (B) Biofilm RB6-4 prélevé en fin de troisième cycle, après l'ajout d'espèces exogènes dans le réacteur RB6. Les trois réacteurs RB6, RB7 et RB8 ont présenté une augmentation de l'épaisseur du biofilm. Le réacteur contrôle RBC n'ayant pas reçu d'espèces exogènes, quant à lui, a présenté une épaisseur constante du biofilm au cours des 3 cycles batch. La formation de bulles de gaz en surface de RB6-4 (B) correspondrait à la production de méthane détectée dans le ciel gazeux.

COUPLING DARK FERMENTATION AND MICROBIAL ELECTROLYSIS FOR HYDROGEN PRODUCTION : FORMATION AND CONSERVATION OF ELECTROACTIVE BIOFILM

Biohydrogen production from dark fermentation is limited because of the associated production of organic acids. These by-products can be used as substrates in a microbial electrolysis cell (MEC), since electroactive biofilms can completely convert organic matter into hydrogen and carbon dioxide. This work aims at analyzing, in the context of the coupling of those two processes and for each one, the relationship between microbial community structures and the associated macroscopic functions. The originality of this study is to work in saline conditions (30-35 gNaCl/L), which favors the charges transfer in the MEC electrolyte but is poorly studied in dark fermentation. Results showed a high selection of microorganisms in both processes associated to good hydrogen production performances. Some of whom are poorly characterized until now. An iron-enrichment method to enrich electroactive bacteria is also proposed. Finally in coupling situation, the introduction of biomass originated from dark fermentation could result to a decrease of biofilm activity.

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