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Eco-Ingénierie des biofilms électro-actifs

Jean-Clément Flayac

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Thèse pour obtenir le grade de Docteur

Délivré par MONTPELLIER SUPAGRO

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Présentée par Jean-Clément FLAYAC

Éco-ingénierie des biofilms électroactifs

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Eco-engineering of electroactive biofilms

Bioelectrochemical systems (BES) such as microbial fuel cells (MFCs) or microbial electrolyzers (MECs) have been the subject of growing interest over the last decade for the production of electricity (MFCs) and, more recently, hydrogen (MECs) or molecules of interest. These processes have in common anodic biofilms, called electroactives, i.e. capable of exchanging electrons with an electrode via extracellular electron transfer mechanisms. The behavior of these biofilms is driven by several interactions within electroactive consortia but also with fermentative bacteria forming a complex trophic network. This PhD thesis aims to understand electroactive ecosystems in order to subsequently implement strategies to improve these consortia and ultimately MXCs. A first step was to understand the interactions within electroactive bacteria. Then, a study established metabolic networks to better understand the relationships between electroactive and fermentative bacteria. Following this, an eco-engineering strategy was tested to improve the performance of anodic consortia. Finally, a study of microbial ecology in real conditions was carried out to set up an effective microbial consortium via inoculation strategies with industrial wastewater as substrate into MFC. This thesis opens new insights in the ecological relationships within electroactive consortia and provide some guidelines to enhance them with the objective of a larger scale application of MXCs.

KEYWORDS: Anodic consortia – Electro-microbiology – Eco-engineering – Ecological relationships

Éco-ingénierie des biofilms électroactifs

Les systèmes bioélectrochimiques (BES) tels que les piles à combustible microbiennes (MFC) ou les électrolyseurs microbiens (MEC) ont fait l'objet d'un intérêt croissant au cours de la dernière décennie pour la production d'électricité (MFC) et, plus récemment, d'hydrogène (MEC) ou de molécules d'intérêt. Ces procédés ont en commun des biofilms anodiques, appelés électroactifs, c'est-à-dire capables d'échanger des électrons avec une électrode par des mécanismes extracellulaires de transferts d'électrons. Le comportement de ces biofilms est déterminé par plusieurs interactions au sein des consortia électroactifs, mais aussi avec des bactéries fermentaires formant un réseau trophique complexe. Cette thèse vise à comprendre les écosystèmes électroactifs afin de mettre en œuvre des stratégies pour améliorer ces consortia et finalement les BESs. Une première étape consistait à comprendre les interactions au sein des bactéries électroactives. Ensuite, une étude a établi des réseaux métaboliques pour mieux comprendre les relations entre les bactéries électroactives et fermentaires. Par la suite, une stratégie d'éco-ingénierie a été testée pour améliorer les performances des consortia anodiques. Enfin, une étude d'écologie microbienne en conditions réelles a été réalisée pour mettre en place un consortium microbien efficace par des stratégies d'inoculation des eaux usées industrielles en tant que substrat en MFC. Cette thèse ouvre de nouvelles perspectives sur les relations écologiques au sein des consortia électroactifs et fournit des lignes directrices pour les améliorer dans l'objectif d'une application à plus grande échelle des BESs.

MOTS-CLÉS: Consortia anodiques – Électro-microbiologie – Éco-ingénierie – Relations écologiques

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List of abbreviations

ANOVA	Analysis of variance
AQDS	Anthraquinone-2,6-disulfonate
BC	Betweenness centrality
BES	Bioelectrochemical system
CD	Current density
CE	Coulombic efficiency
CEA	Cloth electrode assembly
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
DIET	Direct interspecies electron transfer
DNA	Deoxyribonucleic acid
EAB	Electroactive bacterium
EC	Eigenvalue centrality
EET	Extracellular electron transfer
HIT	Hydrogen interspecies transfer
HPLC	High-Performance liquid chromatography
HRT	Hydraulic retention time
IET	Interspecies electron transfer
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
NGS	Next generation sequencing
ORE	Organic removal efficiency
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
P_{drop}^{7d}	Propionate drop during seven days
PTFE	Polytetrafluoroethylene
qPCR	Quantitative real-time polymerase chain reaction
RAP	Redox active protein
rRNA	Ribosomal ribonucleic acid
SCE	Saturated calomel electrode
SHE	Standard hydrogen electrode

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Introduction

L'utilisation massive de carburants fossiles a considérablement augmenté la pollution et provoqué des dérèglements globaux majeurs, induisant une absolue nécessité de produire des énergies renouvelables. De nouvelles solutions doivent être considérées afin de produire un vecteur énergétique propre. Dans ce cadre, l'hydrogène semble une alternative prometteuse notamment dans le domaine du transport. Parmi les technologies capables de produire de l'hydrogène, les cellules à électrolyses microbiennes (MECs) constituent une solution d'avenir [1]. Dans les MECs, la matière organique contenue dans les eaux usées est oxydée à l'anode en CO_2 , électrons et protons par des bactéries spécifiques appelées bactéries électroactives (EABs). Les électrons transférés à l'anode traversent le circuit électrique jusqu'à la cathode où ils réagissent avec les protons pour produire de l'hydrogène. Cette réaction biologiquement assistée requiert une plus faible tension appliquée (0.2-0.8 V) par rapport à l'électrolyse de l'eau (1.23-1.8 V) [2].

Des avancées significatives ont été récemment rapportées dans l'amélioration des performances des MECs, selon deux principaux indices : la densité de courant maximale (CD_{max}) et le rendement Faradique (RF) [3]. L'amélioration de ces deux indices est essentielle dans la perspective d'une application à plus large échelle. Tandis que de nombreux paramètres (architecture du réacteur, matériaux) peuvent impacter les performances des MECs, le biofilm anodique, en tant que catalyseur, est le paramètre fondamental à optimiser pour une conversion

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optimale de la matière organique en électrons [4]. Ces biofilms sont pour la plupart composés d'EABs capables d'utiliser l'anode en tant qu'accepteur terminal d'électrons [5]. Le transfert électronique depuis une cellule bactérienne vers l'anode peut être réalisé soit directement avec des pili conducteurs et des protéines à activité rédox extracellulaires comme des cytochromes C, soit indirectement par des navettes électroniques capables d'être oxydées à l'anode [6]. Pendant l'oxydation des molécules organiques, comme les acides à chaînes courtes, il est nécessaire pour certains acides, comme le propionate ou le butyrate, de maintenir une faible pression partielle d'hydrogène pour rendre l'oxydation thermodynamiquement favorable [7]. Ceci implique une interdépendance obligatoire entre les bactéries productrices et consommatrices d'H₂, appelée syntrophie [8]. Donc, pour la complète conversion de substrats fermentaires comme le glucose, le propionate ou l'éthanol en électrons, les produits fermentaires intermédiaires tels que l'hydrogène, le formiate ou l'acétate sont utilisés par des bactéries électroactives capables de générer un courant électrique. Certaines bactéries électroactives fermentaires comme *Geobacter metallireducens*, peuvent convertir de nombreux substrats en courant sans le besoin de partenaires fermentaires grâce à une très grande versatilité métabolique [9]. D'autres EABs comme *Geobacter sulfurreducens* ont par comparaison une très faible versatilité métabolique et sont incapables d'utiliser directement le butyrate ou le propionate ce qui les rend dépendantes des bactéries fermentaires [10]. Par conséquent, de nombreuses modalités d'interactions semblent possibles au sein des biofilms électroactifs, et peu d'informations sont actuellement disponibles sur ces systèmes.

Résumé

Au cours de cette thèse, des cellules à électrolyses microbiennes ont été dans un premier temps utilisées en batch avec quatre acides à chaîne courte (acétate, lactate, propionate et butyrate) dans le but de lier la structure des communautés microbiennes en fonction du donneur d'électrons. Ces consortia ont aussi été analysés sous l'angle du core-microbiome, c'est à dire des bactéries en commun soit sur l'anode, soit dans le milieu liquide, pour établir un réseau métabolique à partir de ces dernières. Une problématique majeure identifiée lors de cette première expérience a été la persistance du propionate au cours du temps. Pour lever ce problème, une stratégie de pré-acclimatation à base de substrat a été testée consistant à pré-acclimater des anodes avec des substrats simples pour favoriser la présence de bactéries électroactives dites efficaces pour convertir le propionate en courant comme *Geobacter toluenoxydans*, préalablement identifiée dans les consortia anodiques alimentées au propionate. Pour finir, une stratégie d'inoculation, consistant à mélanger des inocula, a été mise en place dans le but d'améliorer le traitement d'une eau usée industrielle en courant dans des piles à combustible microbiennes.

Consortia microbiens anodiques alimentés avec des acides à chaîne courte en électrolyseurs microbiens : la significativité des structures écologiques.

Actuellement, les consortia anodiques associés à un acide à chaîne courte en MECs sont peu caractérisés. Ces acides sont présents en majorité lors du traitement des eaux usées, car ils proviennent des processus fermentaires [7]. Leur conversion en courant est réalisée par de nombreuses espèces microbiennes. L'analyse bibliographique montre qu'une grande diversité

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est communément observée avec une prédominance d'électroactives et de bactéries ferriréductrices. Parmi les électroactives connues, *Geobacter sulfurreducens* est souvent l'EAB prédominante lorsque de l'acétate ou du lactate étaient présents en donneur d'électrons [11, 12]. Concernant le propionate, *Geovibrio ferrireducens* a été détectée en pile à combustible microbienne alimentée au propionate [13]. Enfin, avec du butyrate en donneur d'électrons, *Pelomonas saccharophila* a été observée suggérant son implication dans la conversion de ce substrat en courant [13]. Cette haute complexité est un obstacle dans la compréhension des mécanismes écologiques des consortia électroactifs. Une manière de simplifier le système est de premièrement identifier les EABs associées à un acide organique donné et deuxièmement de mettre en relation l'abondance des bactéries avec les indices de performance : CD_{max} et RF. De nombreuses EABs spécifiques à un substrat peuvent potentiellement améliorer la performance des MECs. Ces bactéries électroactives sont efficaces, car elles contribuent significativement à la conversion de substrats spécifiques en courant [14].

L'objectif de cette étude était d'identifier les consortia microbiens spécifiques à un substrat donné. Les quadruplicats permettent en même temps de mettre en relation les structures des communautés avec les densités de courant. Pour cela, quatre types d'acides à chaîne courte, l'acétate, le lactate, le butyrate et le propionate ont été utilisés séparément en batch et en quadruplicats.

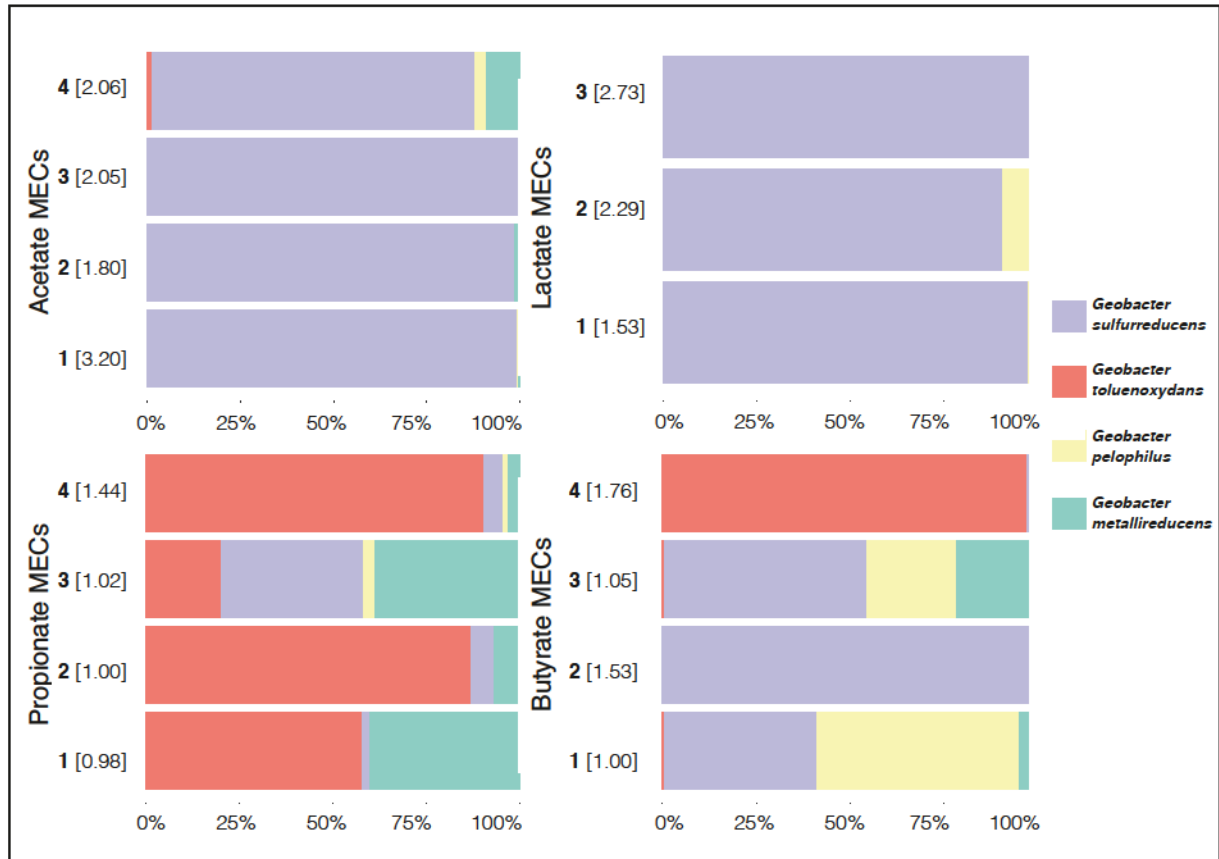


Figure 1 Abondances relatives des *Geobacter sp.* dans les biofilms anodiques des MECs alimentées avec différents substrats. Les séquences de *Geobacter sp.* représentent 62,41% du nombre de séquences totales. Chaque couleur représente une espèce de *Geobacter*. Les nombres (1-4) en gras sont spécifiques au numéro du réplique. Les nombres entre crochets correspondent aux CD_{max} .

Les résultats montrent que *Geobacter sulfurreducens* était majoritaire dans les MECs alimentées à l'acétate, au lactate et deux MECs alimentées au butyrate (2- & 3-Butyrate) (Figure 1). Concernant les MECs alimentées au propionate, *Geobacter toluenoxydans* était prédominante (excepté pour le réplique 3). *Geobacter metallireducens* était la deuxième espèce la plus abondante dans les MECs Propionate-1 & 3 et présente à 9 et 21% dans les MECs

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Acétate-4 et Butyrate-3. *Geobacter pelophilus* était prédominante dans les MECs Butyrate-1 avec une abondance relative de 53%.

De ces résultats, il peut être observé deux structures différentes, la première est basée sur la prédominance de *Geobacter sulfurreducens* capable d'oxyder l'acétate et le lactate à l'anode. Cette dernière semble compétitive pour l'accès à l'anode au détriment des autres bactéries électroactives comme *G. metallireducens* ou *G. pelophilus*. La deuxième structure est basée sur la prédominance de *Geobacter toluenoxydans*, possédant une plus haute versatilité métabolique avec la capacité d'oxyder le propionate et le butyrate.

Dans ces expériences, le lactate est fermenté en acétate et propionate. L'acétate est rapidement oxydé alors que le propionate l'est faiblement au cours du temps. *Geobacter sulfurreducens* étant présente en majorité sur les anodes des MECs alimentées au lactate alors qu'elle est incapable d'oxyder le propionate. Ces données suggèrent la mise en place d'un 'effet barrière' induit par cette bactérie électroactive qui sature rapidement l'anode au détriment d'autres EABs. Il est donc nécessaire d'éviter cet 'effet barrière' par une solution qui favoriserait l'émergence de bactéries électroactives à haute versatilité métabolique, comme *G. toluenoxydans*, afin d'améliorer l'oxydation du propionate.

Identification des microbes clés et des interactions dans les communautés microbiennes électroactives par l'analyse du core-microbiome.

Deuxièmement, les données de l'expérience précédente ont été analysées sous l'angle du core-microbiome, avec pour objectif de réaliser des réseaux métaboliques des bactéries

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communes aux biofilms électroactifs ou aux parties planctoniques dans les MECs. L'identification des espèces du core-microbiome est essentielle pour comprendre l'écologie des consortia microbiens, car ces microorganismes apparaissent dans tous les assemblages associés à un habitat particulier et sont donc probablement essentiels à la fonction de ce type de communauté [15, 16]. Cette étude propose une analyse en cinq étapes : (1) identification des core-OTUs ; (2) affiliation taxonomique et identification des métabolismes potentiels ; (3) corrélations entre les core-OTUs ; (4) corrélations entre les core-OTU et les indices de performance (c'est-à-dire CD_{max} et RF) et enfin (5) établissement d'un réseau métabolique potentiel.

Deux consortia ont été identifiés, le premier est constitué des bactéries communes aux biofilms (core-biofilm) et le second à la partie planctonique (core-planctonique). Le core-biofilm était composé d'une bactérie électroactive (*G. sulfurreducens*), une spécialisée dans le recyclage des acides aminés (*P. benzoelyticum*), une bactérie non classifiée, et *Treponema caldarium*, caractérisée comme homoacétogène. De l'extrait de levure (0.2 g.L^{-1}) était ajouté au milieu, apportant des acides aminés, ce qui pourrait expliquer la présence de *P. benzoelyticum* dans le core-biofilm. Concernant *T. caldarium*, sa présence supposerait la mise en place systématique d'une voie métabolique homoacétogène, utilisant probablement l' H_2 produit au sein du biofilm pour produire de l'acétate. L'acétate pourrait donc être oxydés selon deux chemins métaboliques, soit directement par *G. sulfurreducens*, soit indirectement par une première oxydation en H_2 et CO_2 , puis une phase acétogénèse par *T. caldarium* et enfin une oxydation à l'anode par *G. sulfurreducens* (Figure 2). La voie acétogène étant limitée par une étape obligatoire de baisse en pression partielle d'hydrogène (afin de rendre

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thermodynamiquement favorable l'oxydation de l'acétate), cette dernière pourrait être moins efficace et donc diminuer la densité de courant dans le biofilm. Pour vérifier cette hypothèse, une corrélation a été réalisée entre l'abondance absolue de *T. caldarium* et la CD_{max} . Ces deux variables sont linéairement reliées ($r=-0.95$, $pvalue=2.2.10^{-08}$) indiquant que plus l'abondance de *T. caldarium* augmente, plus la densité de courant diminue. Ainsi, *T. caldarium* semble être un bio-indicateur de CD_{max} robuste au sein des biofilms électroactifs alimentés avec des acides à chaîne courte.

Le second consortium identifié est constitué de huit espèces bactériennes. Parmi elles, cinq espèces spécialisées dans l'oxydation des acides aminés, une bactérie homoacétogène (*Bilophila* sp.) et deux électroactives (*G. sulfurreducens* & *Azonexus caeni*). Des liens de corrélations basés sur les abondances absolues standardisées ont révélé des liens significatifs entre cinq OTUs : trois espèces capables d'oxyder des acides aminés (*Macellibacteroides fermentans*, *Lutispora* sp. et *Paraclostridium benzoelyticum*), *G. sulfurreducens* et *Bilophila* sp. Ces résultats suggèrent de possibles interactions entre ces différentes espèces bactériennes, probablement basées sur la baisse de la pression partielle en hydrogène rendue possible par *Bilophila* sp. Cependant, le fonctionnement des consortia planctoniques en MECs est très peu connu, car dans ces milieux aucun accepteur d'électrons soluble n'est initialement ajouté. La seule possibilité pour les microorganismes serait la synthèse de navettes électroniques redox assurant un cycle d'oxydoréduction entre l'anode (oxydation) et les microorganismes planctoniques (réduction).

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Cette étude permet de comprendre la fonction des hydrogénotrophes homoacétogènes comme ayant un rôle probablement important autant dans les biofilms qu'au niveau planctonique. Une étude plus approfondie de ce groupe fonctionnel semble essentielle pour la compréhension de l'écologie des systèmes électroactifs. Le réseau métabolique hypothétique peut être observé Figure 2.

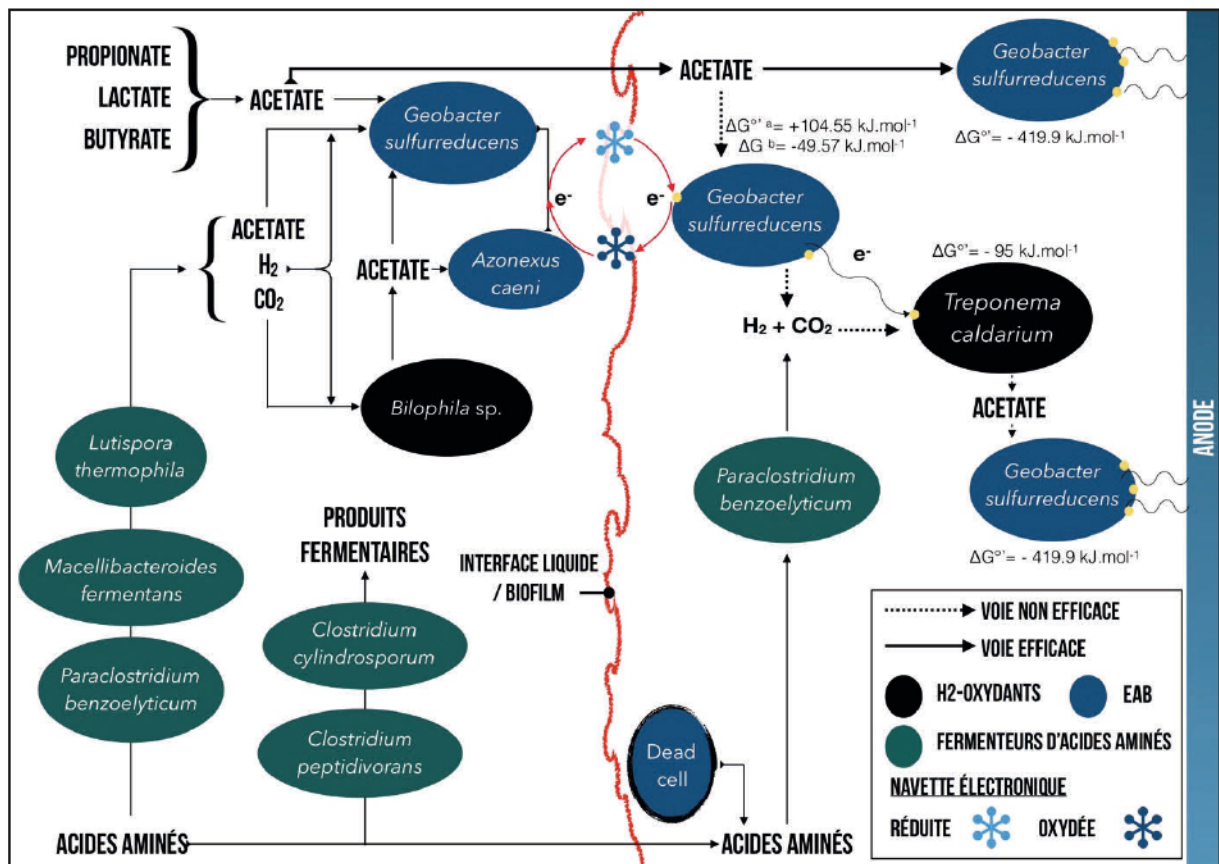


Figure 2 Réseau métabolique hypothétique des bactéries du core-microbiome. Les cercles jaunes représentent les protéines extracellulaires à activité rédox. L'énergie libre de Gibbs change dans des conditions standard (25 °C, concentrations des réactants à 1 M ou 1 atm) à pH 7.0. ΔG^b Calculs des changements d'énergie libre à 25 °C et pH 7.0 selon l'équation $\Delta G' =$

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$\Delta G^{\circ'} + RT \ln([\text{produits}]/[\text{réactants}])$ et sur la base de la concentration suivante : H₂, 10 ppmv à l'état gazeux. E^{o'} (RAP) = 254 mV vs ENH.

Les pré-acclimations anodiques révèlent le rôle essentiel des bactéries sous-dominantes dans la performance des cellules d'électrolyse microbienne.

Le propionate a déjà été décrit comme substrat récalcitrant dans les cellules d'électrolyse microbienne (MECs) [17, 18]. Seules quelques bactéries électroactives sont capables d'utiliser le propionate en tant que source de carbone, comme *Geobacter toluenoxidans*, qui fait concurrence à *Geobacter sulfurreducens* pour la colonisation des anodes. Pour éviter la prédominance de *G. sulfurreducens*, incapable d'oxyder le propionate, l'émergence d'électroactives capables d'oxyder cette molécule pourrait être favorisée par des techniques de pré-acclimation à base de substrats définis. Dans cette étude, des essais de pré-acclimations à base de substrats définis ont été mis en œuvre pour (1) favoriser l'émergence d'EABs capables d'oxyder le propionate et (2) améliorer les performances des MECs alimentées avec du propionate (cinétique d'oxydation propionate, RF et densités de courant maximales). Les résultats montrent que la pré-acclimation des consortia anodiques avec du lactate ou de l'acétate a été plus efficace qu'avec du propionate en améliorant significativement les RF (~9%) de ~75 à ~84%. L'analyse des communautés a révélé que la principale bactérie était *Geobacter anodireducens*, capable d'oxyder directement le propionate en courant (Figure 3).

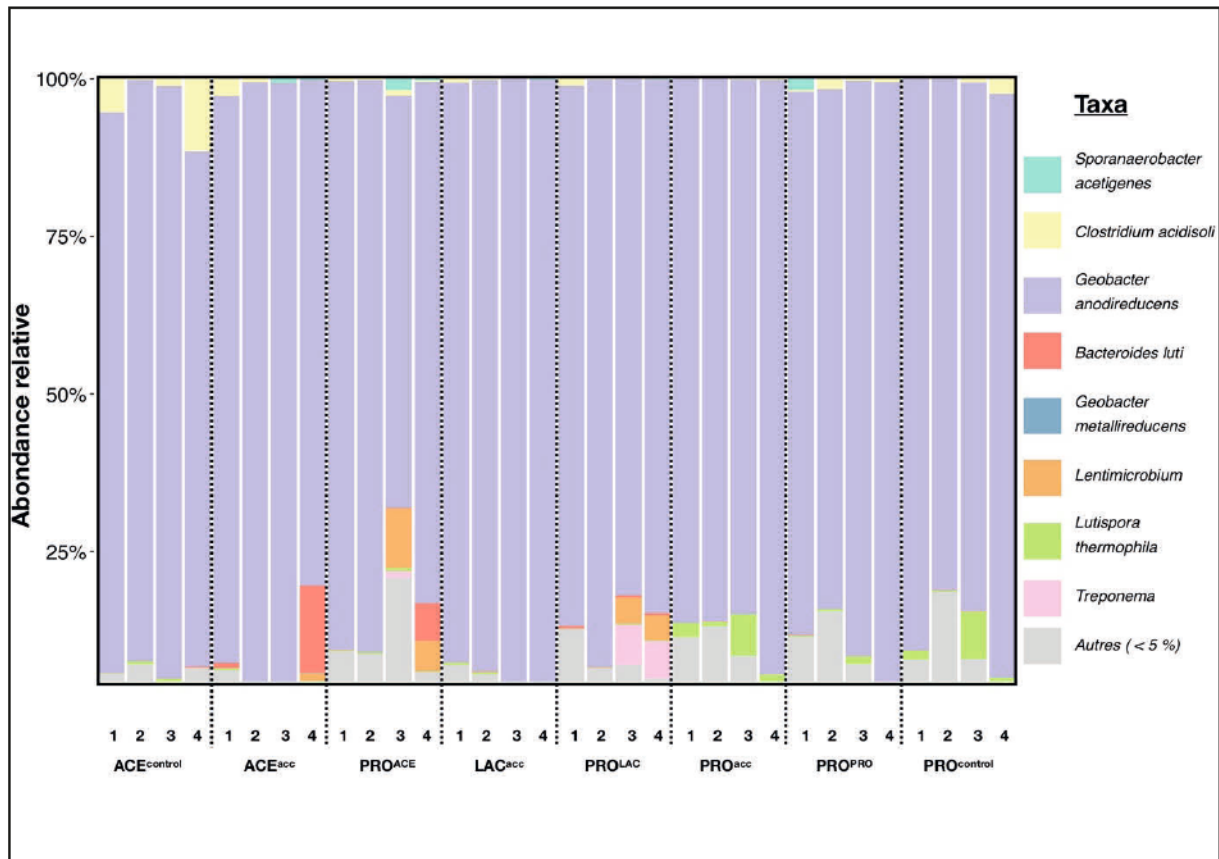


Figure 3 Abondance relative des taxa au niveau de l'espèce dans les biofilms anodiques. Les nombres (1-4) sont spécifiques au réplica. La catégorie 'Autres' représente les OTUs présents à moins de 5% dans l'ensemble des échantillons.

Cependant, cette EAB a été corrélée négativement avec les RF ($r=-0,74$). La pré-acclimation au propionate a favorisée *G. anodireducens* qui a donc diminué les RF contrairement aux conditions pré-acclimatées à l'acétate et au lactate où certaines bactéries peu abondantes ont montré une corrélation avec cet indice de performance. Parmi eux, *Treponema caldarium* était significativement corrélée avec les RF ($r=0,66$). Cette bactérie était précédemment décrite comme homoacétogène et pourrait jouer un rôle dans l'électroactivité du biofilm en oxydant l'hydrogène produit lors de la fermentation du propionate, favorisant ainsi une conversion

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efficace du propionate en courant. Dans l'ensemble, ces résultats suggèrent que les bactéries sous-dominantes pourraient avoir un rôle important dans les biofilms électroactifs et notamment dans l'utilisation efficace de l'hydrogène.

Stratégies d'inoculations pour le traitement d'une eau usée industrielle en pile à combustible microbienne.

Dans le but de développer le consortium microbien le plus efficace pour traiter les eaux usées industrielles, l'utilisation des trois inocula suivant a été mise en place : (1) des boues de stations d'épuration des eaux usées industrielles (2) des boues anaérobies de stations d'épuration d'eaux usées urbaines et (2) une culture enrichie en EABs. Différents mélanges de ces trois inocula et l'utilisation de l'eau usée industrielle sans ajout d'inoculum ont également été évalués. La procédure d'acclimatation a été effectuée en mode MFC. Quatorze MFC identiques équipées d'une cathode à air ont été alimentées avec les eaux usées d'une installation produisant des boissons à base de lait. Selon les différents inocula utilisés, les MFC ont montré des comportements différents en termes d'indices de performance, tels que le rendement Faradique, la densité maximale de courant et l'élimination de la DCO. Les réacteurs inoculés avec des boues industrielles ont surpassé tous les inocula et la combinaison d'inocula testés, produisant une CD_{max} de $0,65 \pm 0,04 \text{ A/m}^2$, avec un RF de $70 \pm 18\%$ et une élimination de la DCO de $77 \pm 10\%$. L'analyse des communautés microbiennes a montré que les MFCs inoculées avec les boues industrielles contenaient plus de *Geobacter* sp. dans les biofilms anodiques par rapport aux autres conditions. *Geobacter* sp. a été corrélée positivement avec l'indice CD_{max} ($r=0,67$)

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et le rendement Faradique ($r=0,73$), ce qui indique son efficacité pour améliorer la performance des MFC avec les eaux usées industrielles. Ces nouvelles connaissances permettent d'envisager des stratégies d'ingénierie écologique en favorisant les *Geobacter* sp. pour le traitement d'eaux usées industrielles.

Conclusions & perspectives

Les résultats rapportés dans cette thèse permettent une meilleure compréhension des consortia électroactifs. En effet, pour la première fois, il a été observé une répartition spécifique des espèces du genre *Geobacter* sp. avec principalement *G. sulfurreducens* concernant l'acétate et le lactate et un mélange entre *G. toluenoxidans*, *G. pelophilus* et *G. metallireducens* avec du propionate et du butyrate. Cette première observation a mis en évidence la présence d'un 'effet barrière' causé par *G. sulfurreducens* qui colonise rapidement l'anode par des processus compétitifs, mais qui est incapable d'oxyder le propionate, diminuant l'oxydation de cette molécule au cours du temps. L'analyse des consortia anodiques a été réalisée de manière complémentaire sous l'angle du core-microbiome, c'est à dire des bactéries en commun soit au niveau planctonique, soit au niveau anodique. Cette analyse a mis en évidence deux consortia, l'un planctonique composé de huit bactéries et l'autre anodique composé de quatre bactéries. L'identification de *Treponema caldarium*, une bactérie hydrogénotrophe résulte de cette analyse. *T. caldarium* était négativement corrélée aux CD_{max} ($r=-0.95$) et une analyse détaillée a montré une probable implication dans la mise en place d'une voie homoacétogène dans les

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consortia électroactifs, moins efficaces pour la conversion des acides à chaînes courtes que l'oxydation directe à l'anode par les EABs.

Les techniques de pré-acclimatation des bioanodes ont montré une amélioration significative des RF. Pour comprendre cette augmentation, des analyses de corrélations ont démontré que cette amélioration en RF était due à l'augmentation de *T. caldarium* et la diminution de *G. anodireducens*. La mise en relation des différentes études montre que *T. caldarium* semble mettre en place une voie homoacétogène dans les biofilms électroactifs, ce qui diminue les CD_{max} (probablement par contrainte thermodynamique), mais parallèlement permettrait une meilleure utilisation de l'hydrogène en évitant les fuites vers des voies concurrentes à l'oxydation anodique.

Les travaux réalisés en MFCs selon différents inocula et de l'eau usée industrielle en substrat ont montré une relation significative avec la présence de *Geobacter* sp. sur les bioanodes et les CD_{max} . Cette avancée est importante pour considérer des techniques d'ingénierie écologique afin d'améliorer les performances des MFCs avec des eaux usées réelles.

Les pré-acclimations anodiques ont montré que lorsqu'une bactérie à haute versatilité métabolique est présente, elle colonise l'anode quel que soit le substrat comme *G. anodireducens*. Par conséquent, l'utilisation du substrat pour pré-acclimater des bio-anodes peut se révéler infructueuse. Afin d'éviter ce problème, il pourrait être envisager des pré-acclimations anodiques avec soit des souches pures, soit des écosystèmes synthétiques qui offriraient à la fois de hautes densités de courant en minimisant l'installation d'une voie

Résumé

homoacétogène et de hauts rendements Faradiques avec une utilisation efficace de l'hydrogène. Une coculture entre *G. anodireducens* et *Hydrogenophaga electricum* semble être une perspective intéressante, avec la première capable d'oxyder de nombreux acides organiques et la seconde capable d'oxyder directement l' H_2 à l'anode sans la mise en place d'une voie homoacétogène.

Une coculture entre *T. caldarium* et *G. sulfurreducens* permettrait de caractériser précisément la relation de ces deux espèces, retrouvées ensemble dans le core-microbiome anodique des MECs alimentées avec des acides à chaîne courte. Une analyse microscopique permettrait de savoir si la voie homoacétogène est mise en place lors de la syntrophie avec *Geobacter sulfurreducens* qui n'est pas directement connecté à l'anode. Il serait intéressant de déterminer si le transfert d'électrons entre ces deux bactéries est direct (pili ou cytochromes de type C) ou médié (par de l'hydrogène).

Ces nouvelles connaissances permettraient une vue plus détaillée des biofilms électroactifs, pour ensuite proposer des solutions efficaces dans la perspective d'une utilisation à plus large échelle des MECs et MFCs.

Short introduction to the manuscript

Bioelectrochemical systems (BES) such as microbial fuel cells (MFCs) or microbial electrolyzers (MECs) have been the subject of growing interest over the past decade for the production of electricity (MFCs) and, more recently, hydrogen (MECs) or molecules of interest. These processes have in common anodic biofilms, so-called electroactive biofilms that are capable of exchanging electrons with an electrode via extracellular electron transfer mechanisms. The behavior of these biofilms is driven by the numerous interactions existing within electroactive consortia but also with fermentative bacteria in the bulk, all forming a complex trophic network. However, due to the high complexity of these ecosystems, these interactions are poorly characterized. Numerous studies have demonstrated a strong link between the structure of the anodic consortia and the efficiency of MXCs (MFC & MEC) in terms of current density (CD_{max}) or coulombic efficiency (CE). From these observations, it seems possible to improve the performance of MXCs by modifying electroactive biofilms through eco-engineering strategies. The work carried out in this thesis aims to better understand electroactive ecosystems and subsequently implement new strategies for improving these consortia and ultimately MXCs. In this sense, the main objectives of this thesis were:

- Identify interactions within electroactive species.
- To provide a better understanding of the interactions that exist between fermentative and electroactive bacteria.

Short introduction to the manuscript

- Implement an eco-engineering strategy to improve the performances of anodic consortia to degrade propionate.
- Understand the ecological interactions of electroactive consortia in the presence of real wastewater.

This manuscript is divided into six chapters: a bibliographical review (Chapter 1), a study of anodic and fermentative consortia associated with a specific electron donor (Chapter 2-3), a study of anodic pre-acclimations (Chapter 4), a study of electroactive consortia associated with the degradation of real wastewater (Chapter 5) and finally the conclusions and perspectives (Chapter 6).

The first chapter of this thesis presents the basics of electroactive biofilms at the cellular level, in pure cultures and mixed cultures. This chapter presents microbial ecology studies performed on different substrates. Eco-engineering strategies, i.e. strategies to achieve a specific result such as high current density generation by modifying an ecosystem, are presented, with a focus on substrate-based pre-acclimation of consortia in order to improve the MXC performance.

The second chapter presents an analysis of the anodic consortia in MECs fed with a specific electron donor: acetate, lactate, butyrate or propionate. These electron donors were selected because they are mainly found in wastewaters. The results show a strong competition to colonize the anode between the four main electroactive bacteria: *Geobacter sulfurreducens*, *Geobacter metallireducens*, *Geobacter pelophilus* and *Geobacter toluenoxydans*.

Short introduction to the manuscript

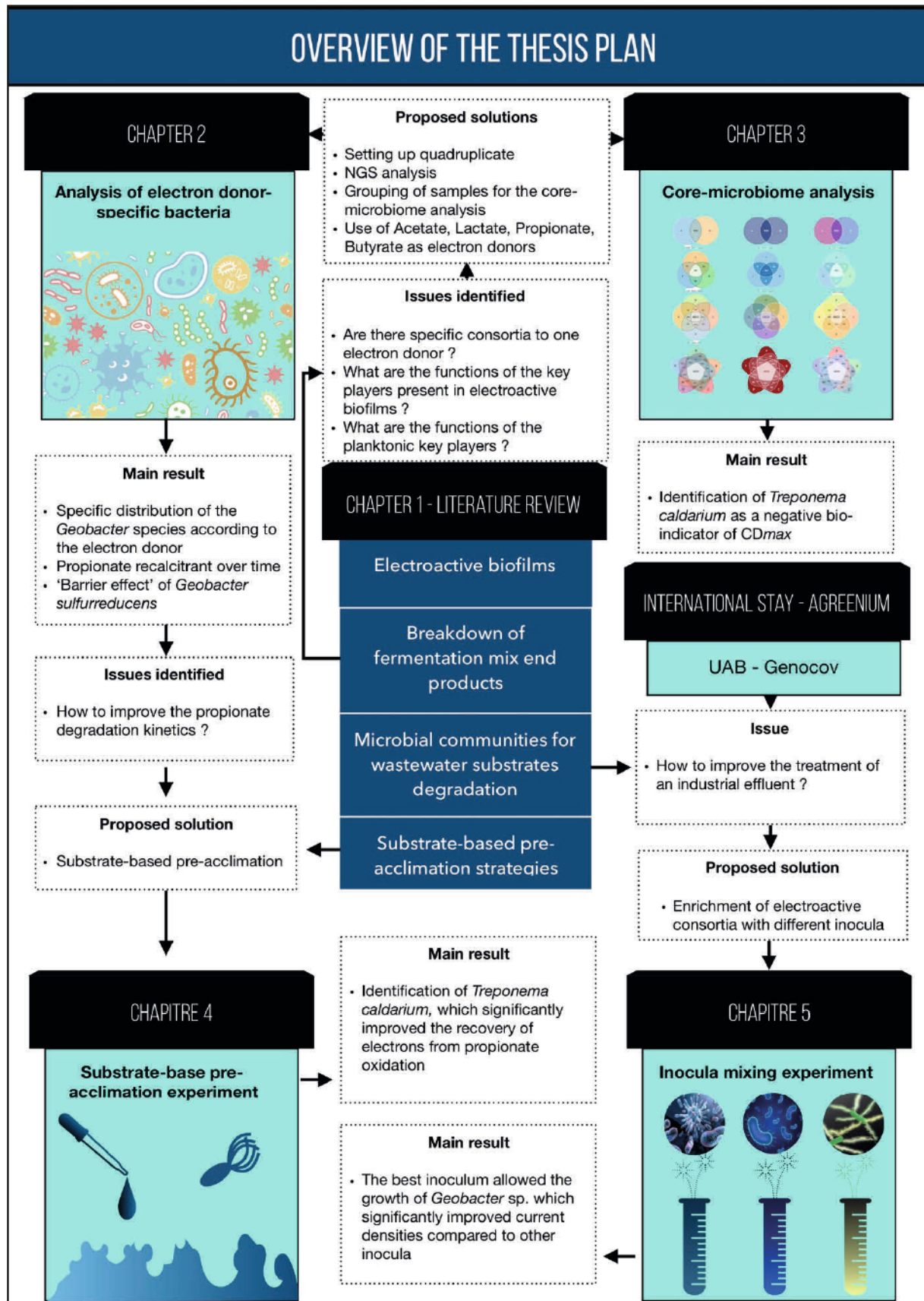
The third chapter presents an analysis of the core-microbiome based on the experience of Chapter 2. The objective was to understand the ecological interactions of the rest of the diversity and planktonic communities. The results show that within electroactive biofilms, an homoacetogenic pathway probably occurred in presence of *Treponema caldarium* in all biofilms.

Chapter 4 presents an eco-engineering study to improve the degradation of propionate into MECs. Bio-anodes were pre-acclimated with different electron donors (acetate, lactate or propionate) and then transferred into a new MEC in presence of propionate. The results show a significant improvement of the CE from ~74% to ~84% with lactate and acetate pre-acclimated consortia compared to propionate pre-acclimated bio-anode. Microbial ecology analysis shows that *Treponema caldarium* significantly contributed to such performance improvement.

Chapter 5 focuses on the study of electroactive consortia in MFCs with real wastewater. With the objective of developing the most effective microbial consortium to treat industrial wastewater, the use of a sludge sampled from industrial wastewater treatment plant as inoculum was compared to an anaerobic sludge from urban wastewater treatment plant, as the most commonly used inoculum in BES, and with an EAB enriched culture. Different mixtures, of the three inocula and the use of the wastewater itself were also evaluated. The results show improved performance due to the inoculation strategy based on the use of sludge from a facility producing a milk-based beverage. The microbial ecology study revealed that the genus *Geobacter* sp. had contributed to this improvement in maximum current density.

Short introduction to the manuscript

Finally, the section 'Conclusion and perspectives' provides a summary of the main results and different ways to understand and improve electroactive biofilms.



The results of this thesis were valorized in the following publications:

- **Flayac JC.**, Trably E., Bernet N. (2018) Microbial Ecology of Anodic Biofilms: From Species Selection to Microbial Interactions. **Published in** *Microbial Fuel Cell – A Bioelectrochemical System that Converts Waste to Watts*, D. Das (ed.), Springer International Publishing, New Dehli, India, pp. 63-85 (doi: 10.10007/978-3-319-66793-5_4).
- **Flayac C.**, Trably E., Bernet N. (2018) Microbial anodic consortia fed with fermentable substrates in microbial electrolysis cells: Significance of microbial structures. Published in *Bioelectrochemistry*, 123, 219-226 (doi: 10.1016/j.bioelechem.2018.05.009).
- Moscoviz R., **Flayac C.**, Desmond-Le Quéméner E., Trably E., Bernet N. (2017) Revealing extracellular electron transfer mediated parasitism: energetic considerations. **Published in** *Scientific Reports*, 7, 7766 (doi: 10.1038/s41598-017-07593-y).

The results obtained in this thesis have also been presented in international conferences as listed below:

- **Flayac JC.**, Trably E., Bernet N. (2017) Microbial Ecology of Anodic Biofilms: From Species Selection to Microbial Interactions. “ISMET 6-2017”, Lisbon (**oral presentation**)
- Desmond-Le Quéméner E, **Flayac JC.**, Trably E., Bernet N. (2018) A core-microbiome approach to identify key microbes and interactions in bioanode microbial communities. “EU-ISMET 2018”, Newcastle (**oral presentation**)

Short introduction to the manuscript

- Marone A., **Flayac C.**, Trably E., Bernet N., Baeza JA., A Guisasola. **(2018)** Start me up! On the choice of a proper inoculum for treating industrial wastewater in BES. “EU-ISMET 2018”, Newcastle **(poster)**

1 Chapter 1 Literature review

1.1 Bioelectrochemical systems

Fundamentals

Bioelectrochemical systems (BESs) are electrochemical devices and therefore consist of an anode and a cathode, immersed in an electrolyte and connected by an external circuit, and typically a membrane separating the two compartments. At the anode, an oxidation process occurs (for example acetate oxidation), whereas a reduction process happens at the cathode (for example O₂ reduction). At least one of these two reactions is catalyzed by an enzyme or a microorganism. BESs are able to convert the chemical energy contained in organic molecules into either electricity (Microbial Fuel Cell or MFC), hydrogen (Microbial Electrolysis Cell or MEC) or other organic products by (bio)electrochemical reduction [2]. From an energetic point of view, it is possible to determine whether a reaction is spontaneous by determining the difference in potential (ΔE) between the two-chemical couples involved (Fig. 1-1). The free enthalpy can be determined according to the following equation ⁽¹⁾:

$$\Delta rG = -n.F. \Delta E \quad (1)$$

Where ΔrG [J. mol⁻¹] is the free enthalpy (or Gibbs free energy), n is the stoichiometric number of electrons exchanged during the reaction, F [C. mol⁻¹] is the Faraday constant and ΔE [V] is

the difference of standard potentials. When $\Delta rG = 0$, the system is in equilibrium; if $\Delta rG < 0$, the reaction is spontaneous and produces energy and is then called exergonic. Finally, if $\Delta rG > 0$, the reaction requires energy input to take place and it is called endergonic. When dealing with MFCs, chemical reactions are spontaneous whereas in MECs, an energy input is necessary to control the anode potential and make the chemical reaction favorable. A wide range of organic substrates are used as electron donors without using precious metals as catalysts in MXCs (MFCs & MECs). On the Fig. 1-1, different electron donors (anode) and electron acceptor (cathode) $\frac{1}{2}$ couples are represented. As described, the reaction is spontaneous when the potential of the electron donor (or reducer) $\frac{1}{2}$ couple is lower than the potential of the electron acceptor (or oxidant) $\frac{1}{2}$ couple. According to all the couples shown in Fig. 1-1, glucose oxidation with oxygen reduction is the most spontaneous reaction, with $\Delta E = 1.23$ V. In this section, MEC and MFC systems will be described.

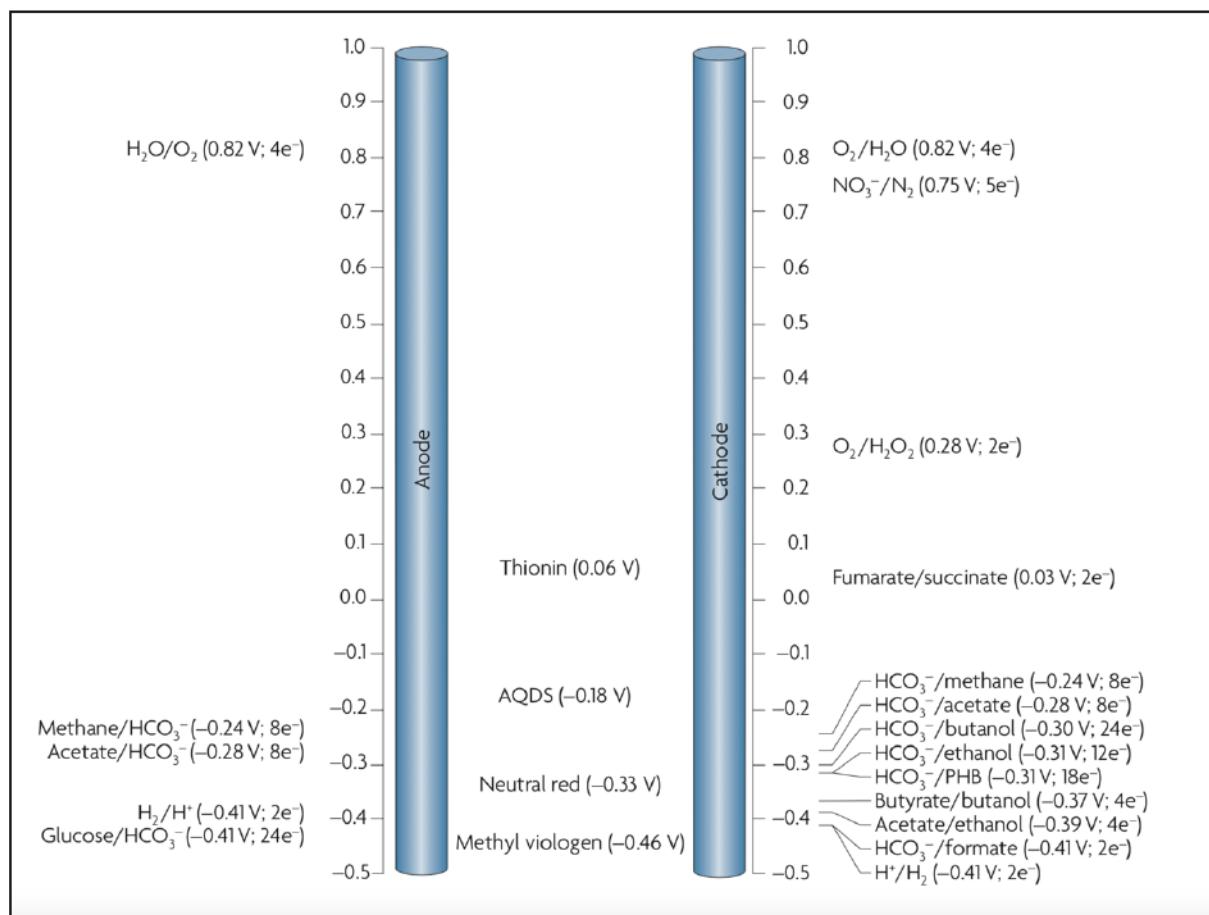


Fig. 1-1 Scale of standard redox potential of specific redox couples. PHB : polyhydroxybutyrate [19].

In 1911, M. C. Potter published his work entitled "Electrical effects accompanying the decomposition of organic compounds". For the first time, a link was established between the degradation of glucose by *Saccharomyces cerevisiae* and the release of an electric current [20]. In 1931, electrical effects were observed during fermentation or putrefaction under the influence of either yeasts or bacteria [21]. This work showed that applying a voltage of 0.5-1 V between the anode and cathode was favorable to the growth of a bacterial culture in a controlled

environment. In the 1960s, there was a renewed interest in using this technology to convert bio-waste into energy in spacecraft [22]. The first patent describing the use of microbial fuel cell was issued to John Davis from Mobil Corporation in 1967, who described an externally mediated MFC using *Nocardia salmonicolor* or environmental bacteria isolated from sludge that oxidized hydrocarbon to alcohols, aldehydes and carboxylic acids [23]. At the end of the last century, in 1999, it was found that MFCs did not necessarily require the addition of chemical mediators to achieve power production [24, 25]. In parallel, another major development was the air-cathode, where no aeration is required [26]. The wastewater treatment and electricity power production was allowed by these two combined technologies.

Two types of MFCs can be distinguished according to the configuration. Either the two chambers (anodic and cathodic) are separated, forming a dual-chamber MFC, or the anodic and cathodic chambers are not separated, thus constituting a single-chamber MFC. In the dual-chamber configuration, the anode and cathode are separated most often by an ion exchange membrane or more rarely by a salt bridge [1]. In single chamber set-up, the most common configuration is the use of an air-cathode, using oxygen as a terminal electron acceptor (Fig. 1-2). However, other acceptors such as nitrate can be used as an alternative [27].

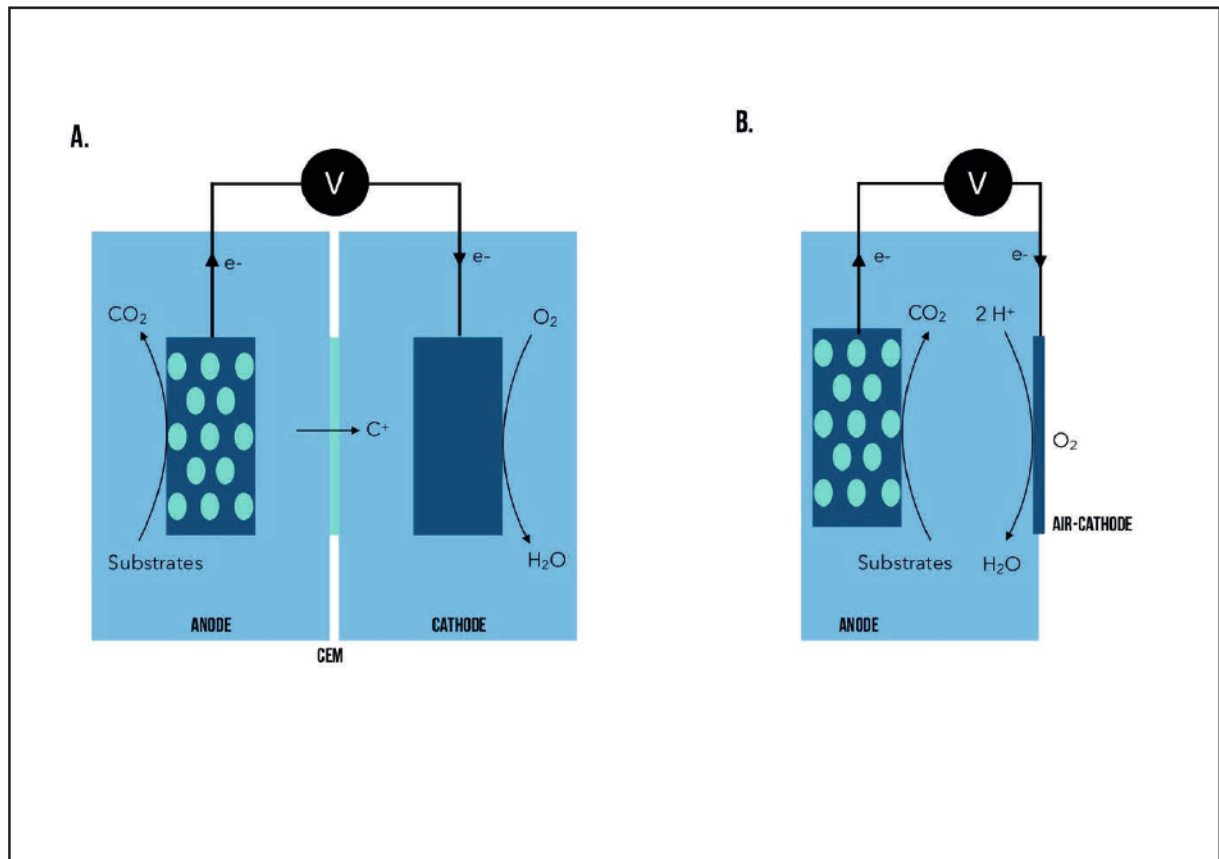


Fig. 1-2 Schematic view of microbial fuel cells. A. Dual-chamber MFC. B. Air-Cathode MFC. CEM = Cation exchange membrane. C^+ = Cation. Anodes are colonized by microorganisms.

In contrast, non-spontaneous reactions occur at the cathode in MECs. Indeed, when a small voltage is applied to the circuit, the production of hydrogen occurs at the cathode by protons reduction (Fig. 1-3). When acetate is used as a substrate, a voltage of 0.13 V is theoretically required for hydrogen evolution, which is significantly lower than the voltage of 1.8-2.0 V used for the hydrogen production by water electrolysis at ambient temperature [28] (Fig. 1-1).

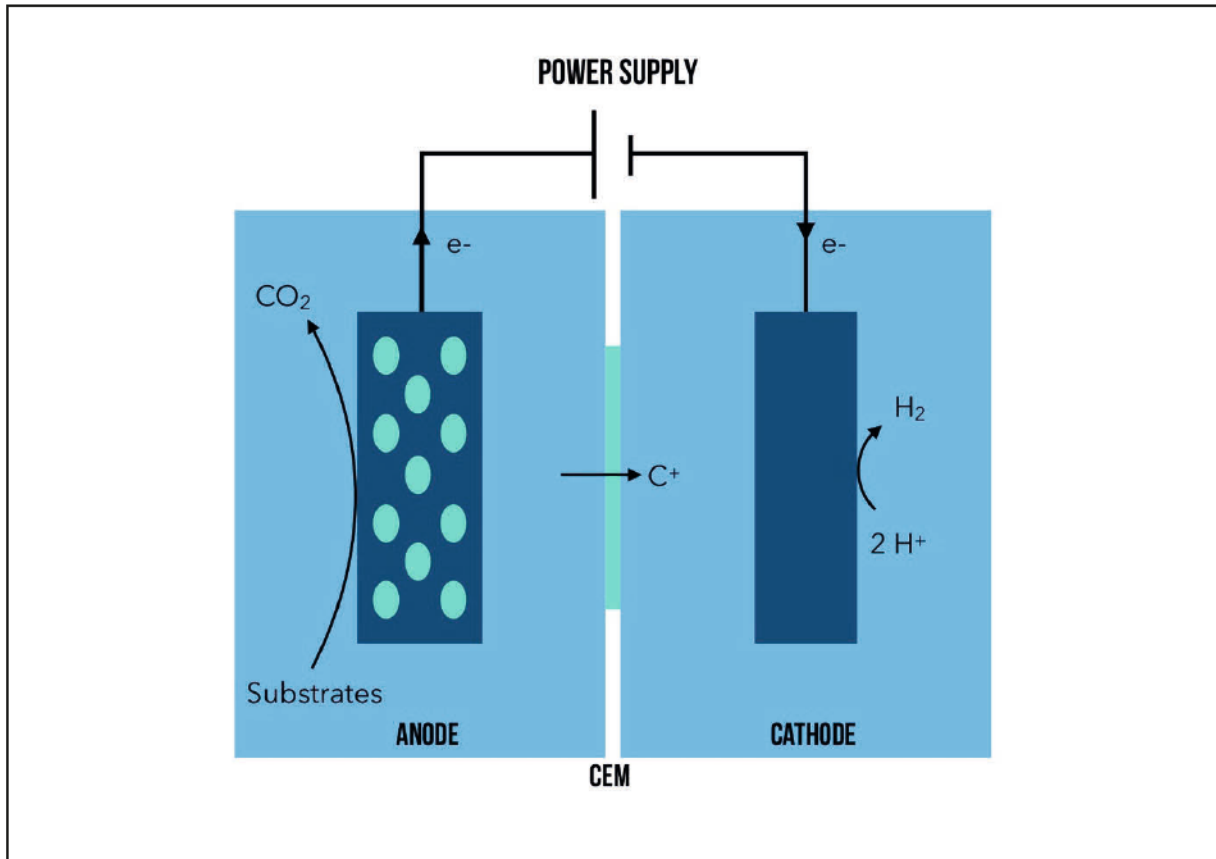


Fig. 1-3 Schematic view of a microbial electrolysis cell. CEM = Cation exchange membrane. C⁺ = Cation. The anode is colonized by microorganisms.

Two main performance indices allow the effectiveness of MXCs to be compared. The first is the current density (or CD). CD is defined as the amount of current flowing through a given cross-sectional area in a given time interval measured in amperes per square meter ($A \cdot m^{-2}$). The current density is directly related to the rate of substrate oxidation by bacteria. Therefore, the maximum current density, or CD_{max} , indicates the highest oxidation rate. The second is the Coulombic efficiency (or CE). CE is defined as the ratio of total Coulombs actually transferred to the anode from the substrate, to maximum possible Coulombs if all substrate removal produced current. The CE unit is the percentage (%). The Coulombic efficiency is

diminished by utilization of alternate electron acceptors by the bacteria, either those present in the medium (or wastewater) [29].

For their operation, MXCs share a common biofilm that develops at the anode, called electroactive biofilm [5]. This structure is studied in a new field called electromicrobiology [30]. In this discipline, the major advance was made in 2002, when it has been demonstrated that microorganisms could oxidize organic compounds to an anode through direct electron transfer. The result was lower operating costs and therefore a hope of using MXCs for wastewater treatment (no addition of chemical mediators was necessary for operation). For this purpose, electroactive biofilms have been studied at many scales, from the ecosystem in the case of mixed cultures biofilms to the bacterial cells and proteins involved in electron transfer to the anode [31]. The mechanisms of electron transfers are described in the following section.

1.2 Electroactive biofilms

Introduction

The electrode, which acts as an infinite electron acceptor, is not an element found in natural environments. By extension, microbe-electrode interactions are thus artificial. The viable microbe-electrode interaction is therefore interesting to explore by identify the evolution mechanisms involved [32]. To interact with an electrode, a bacterium has to externalize the electrons to transfer them to the anode. This mechanism is similar to the respiration of insoluble

metals such as Fe (III) which requires extracellular electron transfer in contrast to other terminal acceptors, for example oxygen, which fully diffuses inside the cell. Even though insoluble minerals and electrodes involve an electron externalization, Fe(III) can be consumed while the anode allows infinite redox reaction. Consequently, the relationship between cell-mineral and cell-electrode involves the same mechanism (outsourcing of electrons) but on two different temporalities, short term for iron reduction and possibly infinite for anode reduction. So, iron reduction requires movement in space, to reach Fe(III) while the reduction of an anode does not require movement, promoting the biofilm lifestyle. For example, in the case of iron reduction, *Geobacter* species are capable of chemotaxis and use their flagella to continuously access to Fe(III) [9]. On the contrary, in contact with an electrode, these cells adhere and form an electroactive biofilm without expression of flagellum [33] (Fig. 1-4).

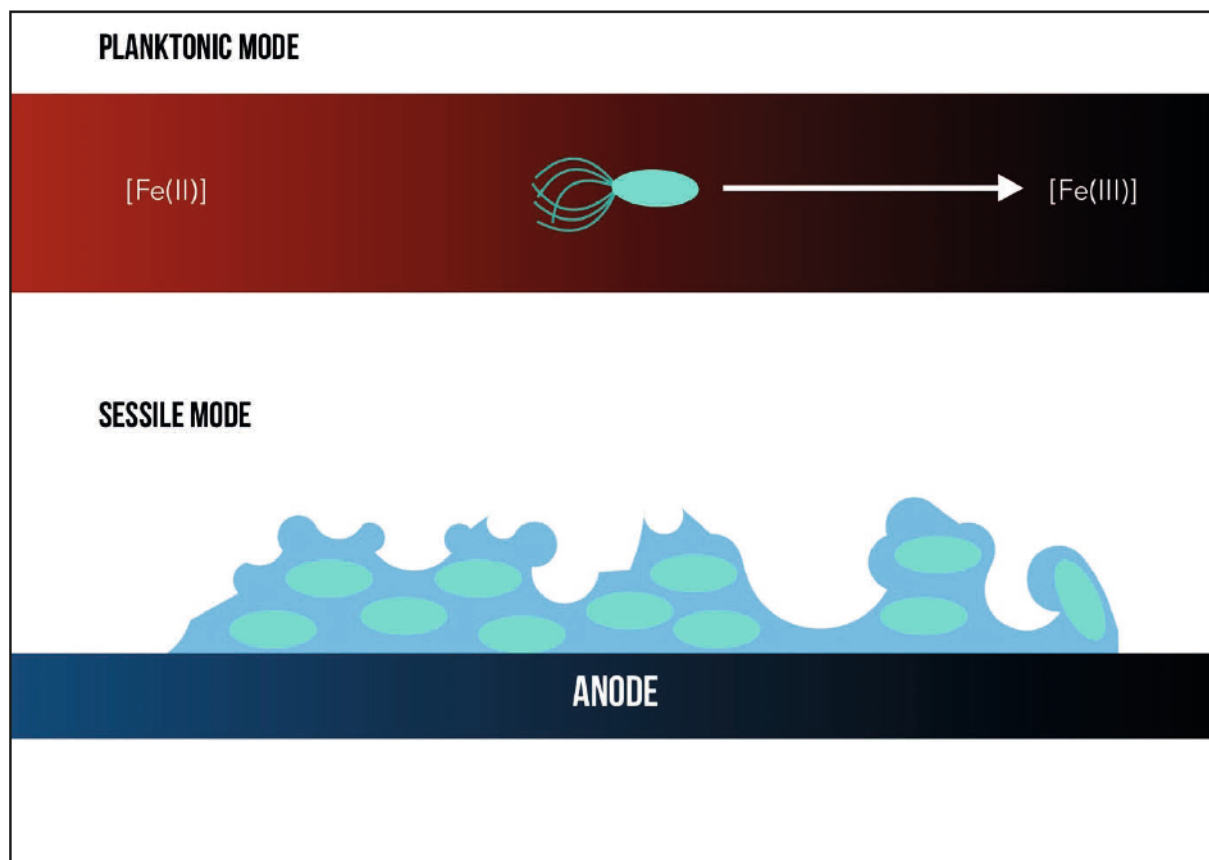


Fig. 1-4 Comparison of the two ways of life of bacteria able to externalize their electrons in a respiratory process. The planktonic mode is found in natural environments while the use of an anode is a mode of artificial respiration.

Three main electron transfer modes between microorganisms and electrodes have been identified. First, indirect electronic transfer: (1) Some microorganisms have the ability to produce electronic shuttles to promote electron transfer to an electrode. The known electronic shuttles are flavins, riboflavins, or polysaccharides. They can be reduced by redox-active proteins on the outer membrane, such as c-type cytochromes. Secondly, short-range direct transfer: (2) Electroactive bacteria can transfer electrons to an electrode by direct contact due to the presence of redox-active proteins such as c-type cytochromes on the outer cell surface.

Finally, long-range direct transfer: (3) the third mode is achieved via conductive pili also called nanowires or e-pili. The direct electron transfer to the electrode is due to the nanowires. Furthermore, they can also rearrange and create electrical networks to make the whole biofilm connected, facilitating inter-species electron transfers [6].

1.2.1 Mediated electron transfer

Some microorganisms can produce electronic shuttles to transfer electrons to the anode. These shuttles are oxidized in contact with the anode and can be reduced by microorganisms in a redox cycle. The model organism for this respiratory strategy is *Shewanella oneidensis*. This planktonic bacterium capable of generating electricity has gained attention with the characterization of flavins as redox molecules in solution. As an illustration, the flavins are reduced by extracellular redox active proteins (RAPs) of *S. oneidensis* (c-type cytochromes) present in the periplasm [34]. Fig. 1-5.C describes this mechanism. The c-type cytochrome MtrC is capable of reducing flavins to the external cell surface, forming part of a multiprotein complex that transports electrons from the periplasm to the external cell surface [35, 36]. Then the electrons are directly transferred from MtrC to the anode [37, 38]. Similarly, MtrC can transfer electrons directly to Fe(III) [39]. However, the current densities produced by mediated electron transfer are lower compared to direct electron transfer due to the low diffusion of the electronic shuttles through the extracellular matrix of the biofilm [40].

1.2.2 Short range electron transfer

Short range electron transfer is achieved by a wide variety of microorganisms. The model organism for this respiratory strategy is *G. sulfurreducens*. Its particularity is this wide variety of c-type cytochromes exposed to the surface of the extracellular membrane (Fig. 1-5.A). Many studies suggested that *Geobacter* species does not use electronic shuttles to reduce Fe(III), nor for electron transfer to the anode. *G. sulfurreducens* has numerous c-type cytochromes exposed on the external cell surface [41-43]. Genetic deletion studies have shown that these same cytochromes permit the electron transfer to extracellular electron acceptors such as Mn(IV), U(VI) or humic acids [44-46].

In the case of *Geobacter* species, extracellular cytochromes function as an electrochemical gate between the cells in contact with the electrode and the electrode surface [47]. A comparison of gene expression via transcriptomics between *G. sulfurreducens* cells producing current versus cells using extracellular electron acceptors (i.e. Fe(III)) made it possible to identify c-type cytochromes that could help *G. sulfurreducens* to establish electrical contacts with an electrode [48, 49]. OmcS appears to be involved in biofilms generating small levels of current while OmcZ appears as the majority cytochrome in biofilms producing high levels of current [5]. Immunogold labeling of electroactive biofilms producing high levels of current have demonstrated that OmcZ had accumulated at the biofilm/anode interface, ideally positioned to facilitate electron transfer to the anode. Thus, OmcZ appears to be a key cytochrome of electron transfer between *G. sulfurreducens* biofilms and anodes [50].

1.2.3 Long-range electrons transfer

The anode, which has a defined surface, is rapidly colonized by electroactive microorganisms. Firstly, they will form a conductive monolayer cell. Then other layers will be added to form a multilayer structure contributing to the production of current densities. To reach the anode when it is not directly accessible, bacteria have developed strategies to externalize the electrons over a long range distance through pili conductors (or e-pili) and conductive filaments [51]. The Fig. 1-5.B summarizes this strategy.

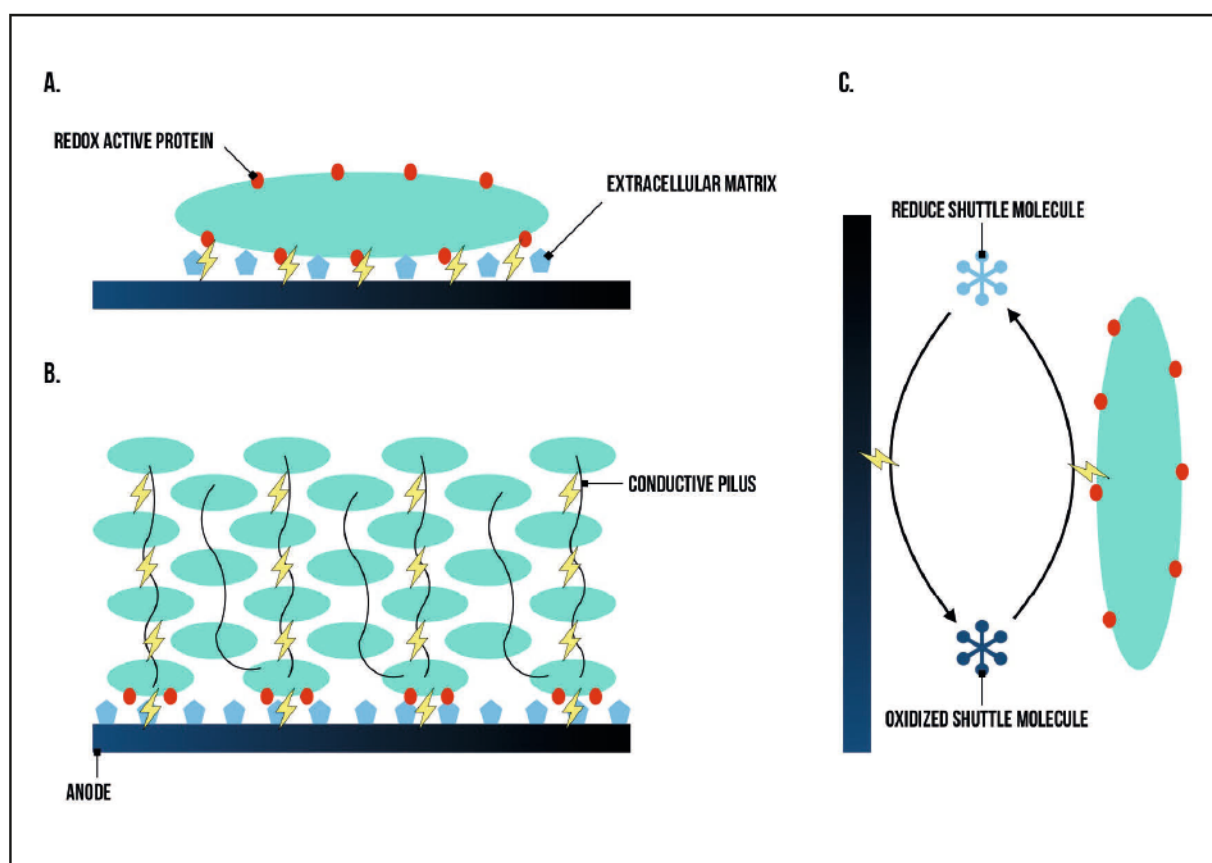


Fig. 1-5 Potential mechanisms for microorganisms to transfer electrons to electrode. A. Short-range electron transfer by microorganisms in close association with the electrode surface through redox active proteins. B. Long-range electron transfer via conductive pili or filaments

accompanied by short-range electron transfer from the biofilm by redox active proteins. C. Electron transfer via reduction of soluble electron shuttles released by the cell. Oxidize shuttle molecule are reduce at the outer cell surface; and the reduce shuttle molecule donate electrons to the anode (Adapted from Lovley. 2012).

1.2.4 Interspecies electron transfer

Biological components allowing extracellular electron transfer such as cytochromes or e-pili are also used during electron transfers between two microbial species. This mechanism is called direct interspecies electron transfer (DIET). DIET is a growth strategy mainly based on syntrophism, in which electrons are transferred from an electron-donating organism to an electron-accepting organism [5]. The connection between the two cells can either be biotic (e.g. e-pili) or abiotic with conductive materials (e.g. biochar or magnetite) [52]. Hydrogen interspecies transfer (HIT) is the alternative strategy in which hydrogen (or formate) is used to transfer electrons between two microbial species. However, for reactions to be spontaneous, it is necessary to decrease the hydrogen partial pressure which decrease the reaction kinetics [53]. DIET has been intensively studied during co-culture between *Geobacter metallireducens* and *G. sulfurreducens* with ethanol as electron donor and fumarate as electron acceptor [54]. *G. metallireducens* can oxidize ethanol but is not capable of reducing fumarate while *G. sulfurreducens* is unable to oxidize ethanol but is capable of reduce fumarate. The co-culture investigation revealed the growth of both bacteria via the establishment of conductive aggregates suggesting electrical connections (e-pili & RAP) in which electrons produced by *G. metallireducens* during ethanol oxidation to acetate were directly transferred to *G. sulfurreducens* which used fumarate as terminal electron acceptor (Fig. 1-6 – A).

DIET can also lead to parasitic interaction. A co-culture, between *G. sulfurreducens* and *Clostridium pasteurianum* was set up with glycerol as electron donor [55]. Only *C. pasteurianum* was capable of fermenting glycerol whose acetate is one of the fermentative products that can be used by *G. sulfurreducens* as an electron donor. However, *G. sulfurreducens* had no electron acceptor in the culture medium but was shown to grow in these conditions. It has been deduced that *G. sulfurreducens* used *C. pasteurianum* as a terminal electron acceptor, probably by reducing the RAPs of the outer membrane of the fermentative bacteria. It was also observed that in the presence of *G. sulfurreducens*, the growth of *C. pasteurianum* was lower than in fermentation alone (Fig. 1-6 – B). To understand the mechanism involved, a thermodynamic study was invested and it was hypothesized that *G. sulfurreducens* decreased the growth of *C. pasteurianum* by electronic parasitism [56].

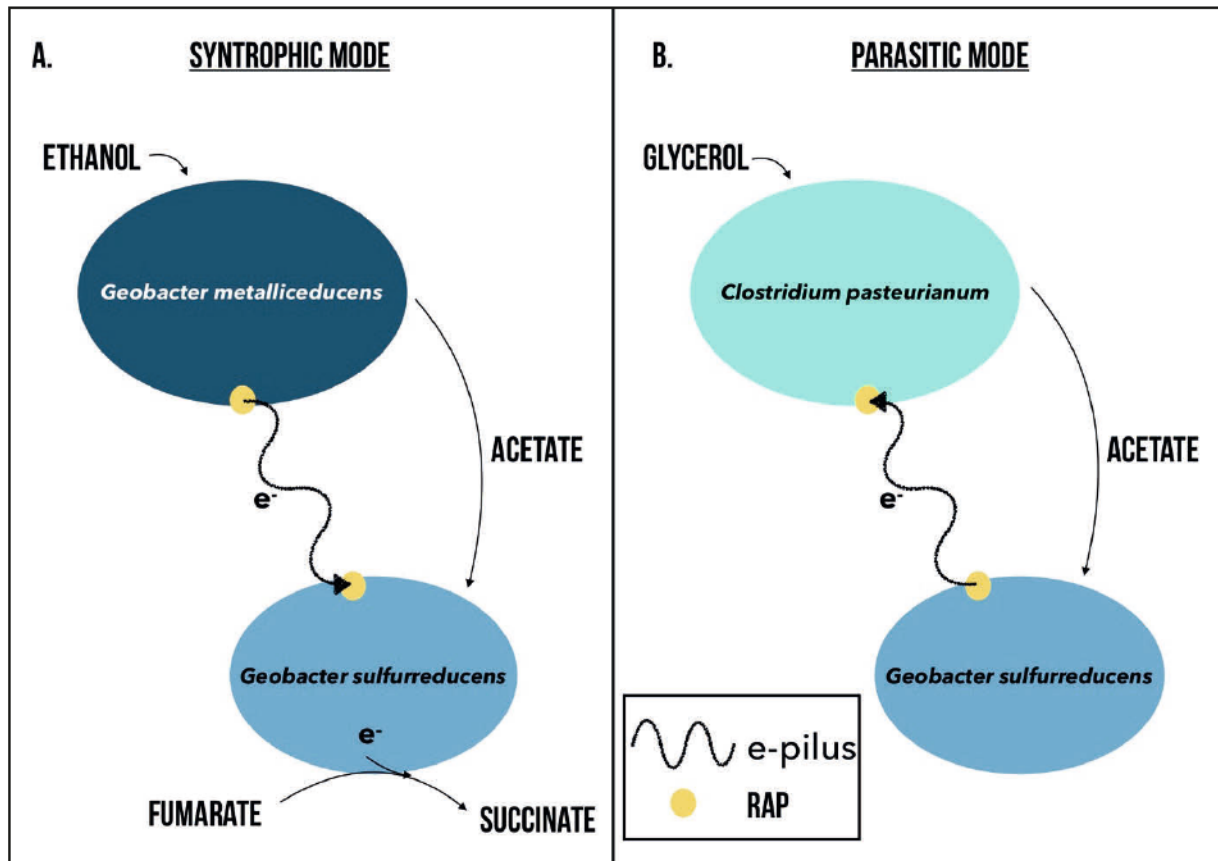


Fig. 1-6 A. Syntrophic-DIET interaction. B. Parasitic-DIET interaction. The glycerol fermentation metabolism is simplified.

The following section has been published as a chapter in the book "Microbial Fuel Cell - A Bioelectrochemical System that Converts Waste to Watts, Das. 2018 - 10.1007/978-3-319-66793-5_4". This chapter presents the various studies carried out studying electroactive biofilms with, on the one hand, pure strain studies to understand the associated metabolism and possibly the electron transfer mechanism, and mixed culture studies to understand the interactions between species according to the electron donor. The chapter first presents the microbial consortia or pure strains associated with the degradation of fermentation end

products, because they are mainly found in wastewaters. For comprehension purposes, these electron donors were used separately, considerably simplifying the ecosystem (Acetate, Formate, Lactate, Propionate, Butyrate and ethanol). Then, the microbial consortia found when glucose was used as fermentation substrate are described, which is an intermediate level of complexity. Even more complex, the microbial consortia found during the use of real wastewater are described.

1.3 Microbial Ecology of Anodic Biofilms: From Species Selection to Microbial Interactions.

Jean-Clément Flayac, Eric Trably, and Nicolas Bernet

1.3.1 Introduction to Electroactive Biofilms

Microorganisms have two main lifestyles: planktonic or sessile. In the planktonic mode, bacteria live in a bulk phase with erratic movements according to hydrodynamics. Advantages of this way of life are the ability to reach new ecological niches and facilitate the access to dissolved substrates. When a planktonic cell attaches to a surface, it becomes sessile. If the bacterial cell multiplies and secretes an exopolymeric matrix on the surface, it forms a structure called biofilm. Biofilms have many advantages including the increase of resistance to antimicrobial agents and the ability of microbes to cooperate for nutrients and/or substrates [57]. According to the type of interactions existing between the microbial biofilms and the

surface, the support can be classified as inert (silica), nutritious (hydrocarbons) or artificial (electrode). Biofilms that develop on conductive materials and exchange electrons with them are called “electroactive biofilms”. In the case of anode-respiring biofilms, *i.e.* biofilms transferring electrons to the conductive material, the terminal electron acceptor is not chemical (O_2 , NO_3^- , Fe(III), Mn(III)) but physical and the bacteria are called electroactive bacteria (EABs).

Three main electron transfer modes between microorganisms and electrodes have been identified: (1) Electroactive bacteria can transfer electrons to an electrode by direct contact due to the presence of redox-active proteins such as c-type cytochromes on the outer cell surface; (2) Some microorganisms have the ability to produce electronic shuttles to promote electron transfer to an electrode. Known electronic shuttles are flavins, riboflavins, or polysaccharides. They can be reduced by redox-active proteins on the outer membrane, such as c-type cytochromes. This is an indirect transfer; (3) Finally, the third mode is achieved via conductive pili also called nanowires. This is a direct transfer to long range. These nanowires allow electron transfer to the electrode. Furthermore, they can also rearrange and create electrical networks to make the whole biofilm connected, facilitating inter-species electron transfers [30].

1.3.2 Breakdown of fermentation mix end products

Commonly, simple organic molecules are generated by fermentative biodegradation of more complex molecules. These by-products can be further oxidized in BioElectrochemical

Systems (BESs) to produce either electricity (MFCs), hydrogen (MECs), or high value molecules formed at the cathode by electrochemical reduction or electro-fermentation [58].

Performances of BESs mainly depend on the type of microbial communities involved in the degradation process. A better understanding of microbial selection and ecological interactions existing between microorganisms is primordial prior to the development of BESs at larger scales. In this part, the community structure of exoelectrogenic microbial consortia fed with different fermentation end products is described here below as well as the syntrophic processes that improve the biodegradation kinetics.

1.3.2.1 Acetate

Acetate is a fermentation end product issued from the acetic acid fermentation. This reaction is carried out by acetic bacteria growing on carbohydrates, primary alcohols, polyhydric alcohols or aldehydes. Acetate is widely used as a model substrate in most lab-scale studies on BESs with pure or mixed cultures. The highest recorded power density obtained with this substrate is 4.3 W m^{-2} , with a novel cloth-electrode assembly (CEA) MFC design [59]. There are two main ways to convert acetate in current, either directly by EABs or through hydrogen production (Fig. 4.1).

The direct way is performed by EABs. Indeed, the acetate oxidation is thermodynamically favourable under standard conditions (Table 4.1). In pure culture, the model bacterium in microbial electrochemistry is *Geobacter sulfurreducens* PCA. *G. sulfurreducens* PCA uses acetate as electron donor and the electrode as electron acceptor [60, 61].

In mixed culture, it is important to determine the carbon flow from the substrate to the ecosystem, to understand the trophic chain. This was achieved by using Stable Isotopic Probing and DGGE in MFC [62]. As expected, *Geobacter* sp. was the most abundant bacteria in the communities (28,3 +/- 7,3%) and was also dominant in heavy fractions indicating that *Geobacter* sp. is a key genus in acetate uptake [62]. Other EABs able to use acetate have also been described in the literature. Most of them are from the *Geobacteraceae* family, including *Geobacter metallireducens*, *G. anodireducens* [63].

The second way for converting acetate to current is through hydrogen production (Table 4.1). Hydrogen scavengers appear to play a key-role in the electron flow from acetate towards anode because access to acetate is limited as compared to dissolved chemical electron acceptors which are present in the solution and easily accessible to all microorganisms. Therefore, close to the anode, acetate oxidation is thermodynamically favourable. When the biofilm becomes thicker, electrode becomes unavailable and hydrogen tends to accumulate, increasing its partial pressure. In this case, *G. sulfurreducens* cannot ferment acetate to H₂ because the reaction becomes endergonic (Table 4.1). This reaction can occur if the hydrogen partial pressure is low enough, *i.e.* in the presence of hydrogen scavengers. Hydrogen scavengers include three different metabolic groups: homo-acetogenic bacteria, hydrogenotrophic methanogens and EABs consuming H₂. Their syntrophic interactions are based on interspecies H₂ transfer [64]. When methanogenesis occurs, hydrogenotrophic methanogens (*Methanobacteriales*) are the H₂ scavengers. On the contrary, when this metabolic group is inhibited, eg. with BES, homo-acetogens (*Acetoanaerobium* sp.) become the main channel for electron flow to convert H₂ to current [65]. Concerning EABs, when hydrogen-consuming partner as *Hydrogenophaga* sp.

AR20 is added, acetate can be oxidized by *G. sulfurreducens* since *Hydrogenophaga sp.* AR20 uses hydrogen via NADH and FADH₂ and transfers its electrons to the anode by long-range electronic shuttle. This example shows the syntrophic cooperation between EABs and hydrogen-consuming partners which appears to be important in these ecosystems where the electron acceptor is solid [66].

In bioelectrochemical systems with mixed microbial cultures, methanogenesis is a strong competitor to current production. Methanogens can indeed metabolize acetate and hydrogen to produce methane, resulting in a trophic competition of EABs towards these two substrates and a strong decrease of the Coulombic efficiency. Basically, trophic competitions in anaerobic mixed cultures (*e.g.* dark fermentation) can be controlled by parameters such as pH or hydraulic retention time (HRT). Regarding BESs, the ability to vary the resistance is an additional way to control competition pathways. To better understand the resistance effects of anodic microbial communities, Jung and Reagan (2011) modified this parameter and measured methane production. In lower external resistance systems, the substrate consumption rate was higher due to increased rates of electrogenesis (327 µeq/day), lower methane production (13 µmol) and higher coulombic efficiency (67%). In contrast, high external resistance induces lower rates of electrogenesis (43 µeq/day), an increase in methanogenic activity (28 µmol of CH₄) and a decrease of the coulombic efficiency (CE 25%). In this study, bacterial 16S rDNA genes were analysed by DGGE. The methanogenic community was dominated by *Methanosaetaceae* (acetoclastic methanogenesis) and *Methanomicrobiales* (hydrogenotrophic methanogenesis), two methanogens families which could interact with EABs to recover electrons (instead of hydrogen) to form methane [67].

1.3.2.2 *Formate*

Formic acid is a short chain organic acid resulting from glucose fermentation. The maximum power density reported with formic acid was 924 mW m^{-2} [68]. Microbial community profiles in MFC powered with formate suggest three different mechanisms to convert this substrate in current: direct conversion by EABs, indirect conversion through homoacetogenesis or hydrogen (Fig. 4.1).

The direct oxidation of formate by EABs is exergonic under standard conditions (Table 4.1). It was observed after addition of an electron-carrying mediator such as humic acids or the humate analogue anthraquinone-2,6-disulfonate (AQDS), by the Gram-positive, spore-forming bacterium *Desulfitobacterium hafniense* DCB2. The generated current reached up to 400 mW m^{-2} of cathodic surface in a single-chamber MFC, with a platinum-containing air-fed cathode [69]. This conversion was also observed without addition of electronic shuttles with *Paracoccus denitrificans* found at 39% abundance in an anodic biofilm of a formate-fed MFC. This bacterium is capable of producing hydrogen from formate and also transfer electrons via flavin shuttle to anode [70].

The second probable way to convert formate to electrical current is a first conversion of formic acid to acetic acid by homoacetogens, an exergonic reaction under standard conditions (Table 4.1). *Acetobacterium sp.* was found at ~25% abundance in anodic communities able to use formate and produce acetate [71]. Therefore, it is an energetically interesting way for microbial

growth. Acetate can be further converted to current by EABs such as *Geobacter sulfurreducens* which represents ~50% of the anodic communities [68].

The third way is the conversion of formate to hydrogen, an endergonic reaction which implies an obligatory syntrophy between microorganisms to keep the hydrogen partial pressure low (Table 4.1). In a formic acid-fed MFC, *Paracoccus denitrificans* dominated the biofilm (26 to 36%), by carrying a formate dehydrogenase that oxidizes formate to carbon dioxide and hydrogen. As hydrogen scavenger, *Geobacter sulfurreducens* converted hydrogen to current and kept the partial pressure of hydrogen very low [68].

1.3.2.3 Lactate

Lactate is a major product of glucose fermentation. Power density of 474 mW m⁻² were obtained in MFC fed with lactate, that is approximately 15% less than using acetate [7]. In BESs, lactate can be converted to electrical current by two main pathways. The first one is the direct conversion by EABs and the second is the lactate fermentation to acetate and propionate that are further used by EABs (Fig. 4.1).

In some studies, pure culture experiments showed the direct oxidation of lactate by EABs without electronic shuttle. The first reaction corresponds to the intracellular conversion of lactate to acetate, followed by acetate and hydrogen oxidation (Table 4.1). The overall reaction is highly exergonic. More particularly, *Geobacter sulfurreducens* PCA oxidizes lactate to pyruvate further converted to acetate that can be oxidized at the anode [72].

The use of mixed culture helped to show the possibility of the second conversion mode through lactate fermentation to current. A one year experiment showed the significant presence of lactic acid fermenters [73]. *Pelobacter propionicus* was found at 39% in an anodic biofilm with lactate as sole carbon source. *P. propionicus* oxidizes lactate in propionate and acetate in a 2:1 molar ratio (Table 4.1). *Geobacter sulfurreducens* was present at 7%, suggesting a fermentation pathway to acetate and consequently a possible syntrophy with *P. propionicus* [73].

1.3.2.4 Propionate

Propionate is a common product in anaerobic ecosystems such as methanogenic systems. Power density produced from propionate is not very high ($\sim 115 \text{ mW m}^{-2}$) in MFC as compared to acetate [74]. Its oxidation involves microbial interactions that are complex and mostly unknown. Based on analysis of microbial communities, three oxidation pathways of propionate were proposed. The electron flow can go directly to the anode with EABs or through a fermentative way depending on the microbial communities present in the electroactive biofilm (Fig. 4.1).

The first degradation pathway is the direct exergonic oxidation of propionate by EABs (Table 4.1). Tests in pure culture revealed that *Geothrix fermentans* is able to oxidize propionate with two electron transfer modes: with or without electronic shuttles. This EAB can oxidize other substrates of interest such as acetate, lactate, malate, and succinate [75].

In mixed culture, DGGE analysis revealed the presence of *Geovibrio ferrireducens* in microbial fuel cell fed with propionate as sole electron donor. This bacterium has c-type cytochromes that suggest a direct flow of electrons to the anode [13].

The second pathway is through a first fermentation of propionate to acetate and hydrogen. However, this reaction is endergonic in standard conditions and a syntrophy between fermenters and hydrogen scavengers is strictly necessary. A bacterial strain with 96% similarity with *Propionibacteriaceae* was identified in a propionate-fed MFC. These bacteria can ferment propionate to acetate [76]. In MEC reactors fed with different propionate concentrations (4.5 mM and 36 mM), *Rhodocyclaceae*, *Clostridiales* and *Bacteroidetes* were the probable propionate fermenters [17]. They were reported to oxidize propionate and were dominant in propionate- and acetate-fed MECs [77-80].

Then, hydrogen can be metabolized by three metabolic groups (Fig. 4.1). Concerning EABs, OTUs close to *Geobacter sulfurreducens* PCA and *G. sulfurreducens* subsp. *ethanolicus* were previously found in propionate-fed MFC and are both capable of using hydrogen to produce current. In addition, only little methane was detected (1.2-2.3%), suggesting that EABs or homo-acetogens were competitive as compared to methanogens. In this study, the major electron sink was the anode (60-80% of total electrons) [17]. The second co-product, acetate, was used more effectively by EABs as compared to methanogens. *G. sulfurreducens* can outcompete acetoclastic methanogens because of their kinetic properties with a K_s at 0.64 mg COD/L versus 177-427 mg COD/L respectively [81]. In addition, *G. sulfurreducens* subsp. *ethanolicus* seems capable of converting hydrogen into electricity. Contrary to the preceding

genus, *Pseudomonas sp.* uses electronic shuttles (pyocyanines) for electron transfer and was already found at 99% similarity with band sequence in DGGE analysis of propionate-MFC [76].

The third pathway is through a first fermentation step to acetate and formate. Propionate oxidation to acetate and formate is endergonic with $\Delta G^{\circ'} = +72.2 \text{ kJ mol}^{-1}$ (Table 4.1). Microorganisms able to achieve this step are identical to those of the second pathway acetate/H₂ (*Propionibacteriaceae*, *Rhodocyclaceae*, *Clostridiales* and *Bacteroidetes*). Similarly, formate oxidation is carried out by the same EABs oxidizing hydrogen (*G. sulfurreducens* PCA, *G. sulfurreducens* subsp. *ethanolicus*) [17].

It is not yet established which is the preferred fermentation pathway (acetate/H₂ or acetate/formate). In anaerobic digestion of propionate, interspecies electron transfer through formate was described as the main mechanism and another study suggested that hydrogen transfer is the preferable way in propionate degrading consortia [82, 83]. Another hypothesis is that hydrogen transfer is efficient at short distance as compared to formate which is dominant way to inter-species electron transfer over a long distance [84]. More recently, biochemical and genome analysis of pure culture of syntrophic volatile acid showed that degraders use electron transfer via hydrogen and formate, simultaneously [85]. An important parameter which influences the production of hydrogen or formate is the pH. Indeed, the shift from formate to hydrogen during glucose fermentation was shown to be mainly due to a decrease of the pH [86]. Formate conversion to hydrogen is endergonic ($\Delta G^{\circ'} = +1.3 \text{ kJ mol}^{-1}$) but very close to the thermodynamic equilibrium (Table 4.1). This reaction is catalyzed by the formate hydrogen lyase complex that is reversible. At 25°C, the pK_a of carbonate is 6.37, so it is stable above this

value and inhibit formate splitting into carbonate and hydrogen [87]. It is possible that pH has also an influence on the choice of propionate degradation pathway with the way acetate/H₂ when pH medium is below 6.37 and acetate/formate when pH is above. Therefore, pH changes influence the fermentation products from propionate and may also influence certain EABs (hydrogenotrophic or formate oxidizers) and finally on the reactor performances.

Thus, electron transfer mechanisms in propionate fermentation are poorly understood. A better knowledge of propionate fermenters and their interactions with EABs could help to understand the laws governing the electron flow from propionate in BESs.

1.3.2.5 Butyrate

Butyrate is one of the main compounds generated during the fermentation of organic matter in anoxic environments [88]. In such environment, further butyrate degradation is performed by syntrophic interactions of butyrate-oxidizing bacteria and hydrogen-scavengers because of thermodynamic limitations in standard conditions [89]. In BESs, the power produced by butyrate (1000 mg L⁻¹, 305 mW m⁻²) was 66% lower than that fed with acetate (800 mg L⁻¹, 506 mW m⁻²) [90]. The microbial ecology studies in butyrate-fed BESs operated with mixed cultures reveal only one major pathway for complete oxidation of butyrate to current with a first fermentation step and a second one that converts metabolic end-products into current (Fig. 4.1).

The first step is the butyrate oxidation to acetate and hydrogen. This reaction is endergonic with $\Delta G^{\circ} = + 48.1 \text{ kJ mol}^{-1}$ and involves syntrophic interactions to decrease the hydrogen partial

pressure and make the reaction possible (Table.1). The most abundant fermenter is *Pelomonas saccharophila*, found as a major band in DGGE analysis from MFC. This bacteria has a high metabolic versatility, may use more than 40 carbon sources and appears to be involved in butyrate oxidation [13, 91].

The second step is the oxidation of acetate and hydrogen to current. These two reactions are thermodynamically favourable and can be performed by *Geobacter sulfurreducens* PCA present at 13.6% in anodic biofilm of butyrate-fed MFC. Organisms from the *Geobacteraceae* family were also found in MEC (9.1%). It seems that these EABs can grow in syntrophy with butyrate oxidizers. Their relative low abundance could be due to the presence of methanogenic competitors (54%), explaining the low coulombic efficiencies of BESs powered with butyrate [92, 93].

As observed in different studies, the conversion of butyrate into current is poorly efficient with only ~10% of the electrons recovered. Methanogenesis seems to be the main explanation for this loss of efficiency. Strategies should be found to inhibit this electron flux towards methane and increase the syntrophy between butyrate-oxidizers and exoelectrogenic hydrogen-scavengers, which should result in redirecting the electron flow to current.

1.3.2.6 Ethanol

Ethanol is a common product of glucose fermentation at neutral pH and is a key breakdown product of cellulose fermentation [86, 94]. Power density produced from ethanol is ~800 mW m⁻² in MFC, about five time less compared to acetate [7]. Mixed culture experiments

reveal that ethanol is first fermented to acetate in BESs (Figure 4.1). This reaction is not thermodynamically favourable with $\Delta G^{\circ'} = +9.6 \text{ kJ mol}^{-1}$ which implies the presence of hydrogen scavengers (Table 4.1). *Pelobacter* species could be involved in this reaction. According to community profiles, *Pelobacter* sp. was present at 85-98% in solution and 35-43% in electroactive biofilm of microbial electrolysis cells fed with ethanol [95].

Furthermore, current was produced in a MFC using a coculture of *P. carbinolicus* and *G. sulfurreducens* with ethanol as the fuel. Basically, *P. carbinolicus* can oxidize ethanol but is unable to product current. *G. sulfurreducens* alone cannot metabolize ethanol but oxidizes acetate and hydrogen from ethanol oxidation by *P. carbinolicus*, utilizing the anode as an electron acceptor. In this syntrophy up to 83% of the electrons available in ethanol were collected as electricity and acetate [96].

Acetate is then converted to current. Among the *Geobacteraceae* family, *G. metallireducens* was found in marine sediments fuel cell and can directly oxidize ethanol to current via c-type cytochromes but are unable to oxide hydrogen [31, 97]. EABs other than *Geobacter* sp. can interact with *Pelobacter propionicus* to convert acetate to current as *Rhodopseudomonas palustris* found at 27% on anode biofilm (with 38% of *Geobacter* sp.) [7]. This purple non-sulfur photosynthetic bacterium could generate high current densities (2.2 W.m^{-2}) without light and transfer electron through anode via c-type cytochromes [98].

Table 1-1 Summary of biochemical reactions during mix end products degradation into BESs. The Gibbs free energy depends on the anodic potential; here we reported values for a near-optimum anodic potential of +230 mV vs SHE.

Substrates	Products	$\Delta G^{\circ'}$ (kJ mol ⁻¹)	References
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Acetate + 4 H ₂ O	2 HCO ₃ ⁻ + 9 H ⁺ + 8 e ⁻	-35.5	(Hari <i>et al.</i> 2016)
Acetate + 4 H ₂ O	HCO ₃ ⁻ + 4 H ₂ + H ⁺	+104.6	(Thauer <i>et al.</i> 1977)
Formate + H ₂ O	HCO ₃ ⁻ + 2 H ⁺ + 2 e ⁻	-49.6	(Hari <i>et al.</i> 2016)
Formate + H ₂ O	HCO ₃ ⁻ + H ₂	+1.3	(Sun <i>et al.</i> 2012)
Lactate + H ₂ O	Acetate + CO ₂ + 2 H ₂	-8.8	(Pankhania <i>et al.</i> 1988)
4 Formate + H ⁺	Acetate + 2 HCO ₃	-99.1	(Hari <i>et al.</i> 2016)
3 Lactate	2 Propionate + Acetate + HCO ₃ ⁻ + H ⁺	-164.8	[99]
Propionate + 5 H ₂ O	2 CO ₂ + HCO ₃ ⁻ + 14 H ⁺ + 14 e ⁻	-72.95	(Hari <i>et al.</i> 2016)
Propionate + 3 H ₂ O	Acetate + H ⁺ + HCO ₃ ⁻ + 3 H ₂	+76.1	(Oh & Logan 2005)
H ₂	2 H ⁺ + 2 e ⁻	-34.9	(Hari <i>et al.</i> 2016)
Propionate + 2 HCO ₃ ⁻	Acetate + 3 Formate + H ⁺	+72.2	(Hari <i>et al.</i> 2016)
Butyrate + 2 H ₂ O	2 Acetate + H ⁺ + 2 H ₂	+48.1	(Thauer <i>et al.</i> 1977)
Ethanol + H ₂ O	Acetate + H ⁺ + 2 H ₂	+9.6	(Thauer <i>et al.</i> 1977)

Hydrogen produced from ethanol fermentation can be used by three metabolic groups as described Fig. 4.1). Therefore, when ethanol is the only electron donor in BES, they are a three-way syntrophy among ethanol fermenters, acetate-oxidizing anode-respiring bacteria and H₂ scavengers. With this information, it would be interesting to find solution in order to promote homo-acetogenic bacteria against methanogenic archaea to increase the coulombic efficiency [95]. For example, it is possible to oxidize ethanol with co-culture without hydrogen production in micro-electrolysis cell. *Acetobacter aceti* oxidizes ethanol to acetate and uses electronic shuttles (Ferrocyanide) to regenerate NADH in NAD⁺ instead of producing hydrogen. Acetate is then oxidized in carbon dioxide by *Escherichia coli* salting-out its electrons by the same electronic shuttle than *A. aceti*. This method enables high coulombic efficiency 87% (10.4 electrons out of 12 from ethanol molecule converted in current) but require artificial electronic shuttles. This cascade reaction through two bacteria can be an effective ethanol-converting path

in current without H₂ production, thus avoiding competition with H₂-oxidizing methanogens but with addition of artificial electronic shuttle [100].

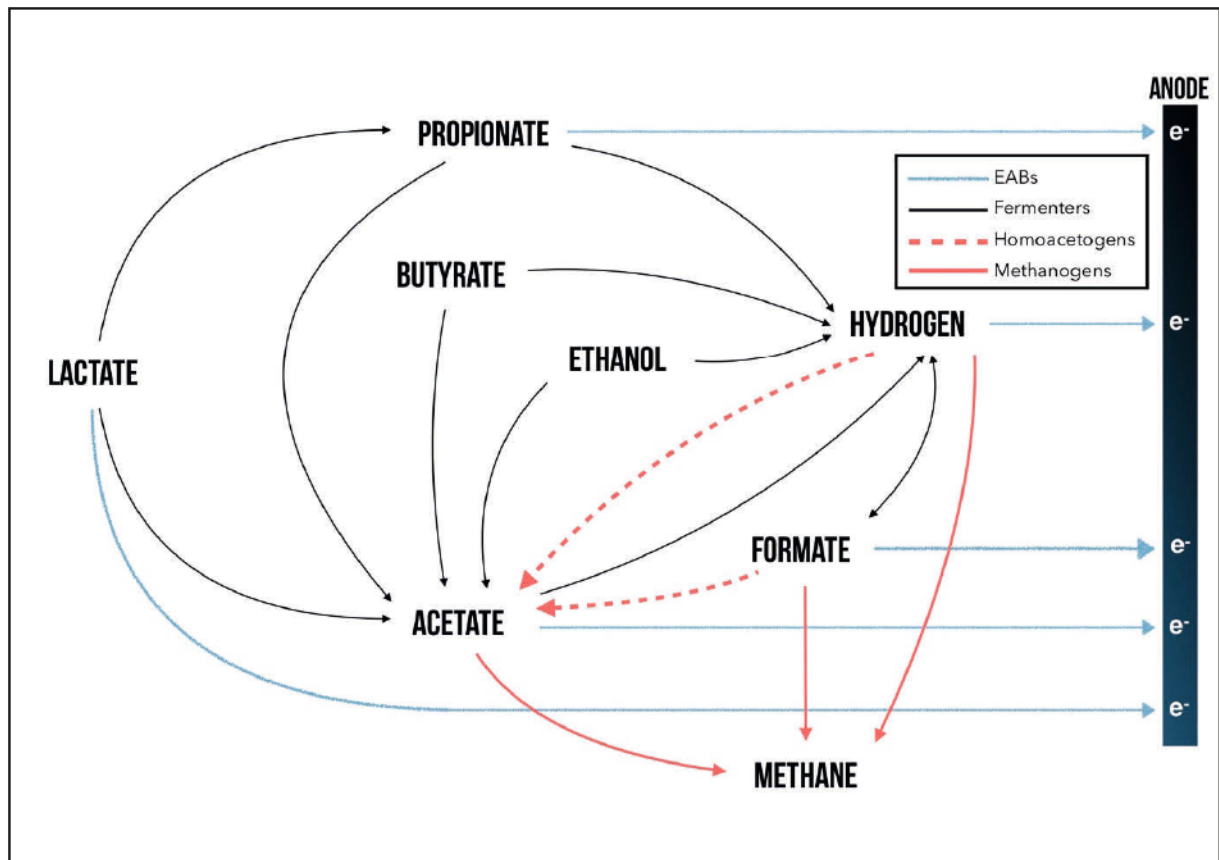


Fig. 1-7 Possible theoretical pathways of electron flow in the anode compartment of BESs from different fermentation end products.

1.3.3 Breakdown of glucose

Conversion of glucose to current is an inefficient process regarding coulombic efficiency as compared to the substrates with lower molecular weight. It is probably due to fermentable nature of this substrate [101]. For example, in mixed culture, the coulombic

efficiency is ~4.8 times lower with glucose as compared to acetate in MFC [92]. Indeed, glucose may be oxidized to many different molecules, which engages competitive processes between microorganisms for obtaining these products (Table 4.2). EABs producing high current densities have limited metabolic versatility, which could prevent them from converting all the fermentation products from glucose to current and allow the possibility of producing methane, the main concurrent route by diverting the electrons flow [102]. Microbial ecology studies provide insight into different possible ways of transferring glucose electrons to the anode. All mentioned microorganisms were observed in glucose-fed BESs. This conversion seems to take place in two major steps: fermentation of glucose to pyruvate (glycolysis) then mix of end product and conversion of these compounds in current with EABs through syntrophic interactions [103].

Table 1-2 Summary of biochemical reactions during glucose degradation into BESs. Concerning half reaction, the Gibbs free energy depends on the anodic potential; here we reported value for a near-optimum anodic potential of +200 mV vs SHE.

Substrates	Products	ΔG° (kJ.mol ⁻¹)	References
Glucose + 6 H ₂ O	6 CO ₂ + 24 H ⁺ + 24 e ⁻	-1438	(Freguia <i>et al.</i> 2008)
Glucose + 2 H ₂ O	2 Acetate + 2 CO ₂ + 4 H ₂ + 3H ⁺	-216	(Freguia <i>et al.</i> 2008)
Glucose	2 Pyruvate + 2 H ₂ + 2 H ⁺	-112.1	[104]
Glucose	2 Lactate + 2 H ⁺	-197	(Freguia <i>et al.</i> 2008)
Pyruvate + H ₂	Lactate	-43.2	[105]
Pyruvate + H ₂	Propionate + H ₂ O	-123	(Thauer <i>et al.</i> 1977)
Glucose	2 Ethanol + 2 CO ₂	-235	(Freguia <i>et al.</i> 2008)

1.3.3.1 Direct conversion of glucose to current

The most direct path is the conversion of glucose into current (Table.2). This reaction releases a large amount of energy with $\Delta G^{\circ} = -1438 \text{ kJ mol}^{-1}$ with anodic potential of +200 mV vs SHE [106].

Electron transfer from glucose to anode can be achieved through membrane proteins. *Rhodospirillum rubrum* carries out this transfer and does not need an electron-shuttling mediator to get energy from oxidation process itself during long-term production [107]. This direct transfer is also made by *Aeromonas* sp. Highly dominant in anodic biofilms (~51.6 %), this genus has electroactive bacteria. In the same conditions but with open-circuit MFC, the abundance of this bacterium decreases to 10 times, suggesting that the extracellular electron transfer is the main metabolism employed and gives to *Aeromonas* sp. an ecological advantage to dominate all other species in this ecosystem. The isolated strain ISO2-3 could ferment glucose and likely transfer electron to the anode through membrane-associated compounds (most likely c-type cytochromes) which requires intimate contact with the anode surface. ISO2-3 can also produce anchor-like filamentous appendages (probably pili) allowing a long-range electron transfer across the thick biofilm [108].

Unlike previous EABs, other bacteria are using electronic shuttles to utilize anode as terminal electron acceptor. Electronic shuttles of *Klebsiella* genus have been well characterized in glucose-fed MFC. Among this genus two species and one strain are able to oxidize glucose to current: *K. pneumoniae*, *K. oxytoca* and *Klebsiella* sp. ME17. The first one was characterized in MFC where the anode was coated with microfiltration membrane (0.22 μm) to eliminate biofilm influence mechanisms in order to better understand electron transfer. The maximum voltage outputs achieved were 316.2 and 427.2 mV after 270 and 120 h, respectively. Cyclic

voltammetry measurements demonstrated the presence of an electronic shuttle produced by *K. pneumoniae* L17 identified to be 2,6-di-tert-butyl-p-benzoquinon (2,6-DTBBQ), as a recyclable mediator able to transfer electrons between *K. pneumoniae* L17 and the electrode [109]. *K. pneumoniae* was found in BESs operated with mixed culture at ~6.45% and 0.02%, suggesting that it might have an interesting role in the efficient conversion of glucose [108, 110]. The second, *K. oxytoca*, was found in glucose-fed MFC at ~7.52 % whereas it was only at ~3.15% in open-circuit MFC (without electron flux). This result suggests that *K. oxytoca* is favoured by the extracellular electron transfer mechanism. *Klebsiella sp.* ME17 was used in an H-type MFC with glucose as electron donor. The maximum power density produced by this strain was 1.209 mW m⁻² with 340 and 1.47 mA of maximum current. The polarization curves illustrated that the strain produced electronic shuttles to promote extracellular electron transfer. Based on cyclic voltammogram, the supernatant was very likely to contain quinone-like substances. However, even after replacing the medium, current was always produced, suggesting that *Klebsiella sp.* ME17 could also transfer electrons through direct contact with electrode [111].

In addition, some EABs use exogenous chemicals mediators to convert glucose to current such as *Shewanella* genus. As an illustration, *S. algae* was observed in MFC exposed to light and can use glucose, ribose and fructose as carbon and energy sources [112]. It is a facultative anaerobe able to produce hydrogen sulfide and reduce Fe(III), making it very corrosive on metal surface [113]. The iron reduction feature and membership in the *Shewanella* genus known to be exoelectrogen, suggests that *S. algae* could be an exoelectrogen whose function is the conversion of glucose to current [112]. Similarly, *Lactococcus lactis* uses exogenous mediators but does not convert all glucose electrons into current, but also in acetate.

This Gram positive bacterium is normally homolactic fermenter able to produce several kinds of membrane associated quinones and to mediate electron transfer with extracellular electron acceptors such as Fe(III), Cu²⁺ and hexacyanoferrate. *L. lactis* can also perform extracellular electron transfer to anode with two soluble redox mediators suggested to be 2-amino-3-dicarboxy-1,4-naphthoquinone (ACNQ). In classic fermentation, *L. lactis* performs the homolactic metabolism while during electro-fermentation acetate and pyruvate are excreted in stoichiometric amounts with respect to the electrical current. This is the first Gram positive bacteria described as capable of extracellular electron transfer [114].

1.3.3.2 Glucose fermentation to mixed end products

1.3.3.2.1 Glucose to acetate and hydrogen

In BES, glucose can be first fermented into acetate and hydrogen. This reaction is thermodynamically favourable with $\Delta G^{\circ} = -216 \text{ kJ mol}^{-1}$ and constitutes the main route of fermentation (Table 4.2). A clone close to *Acetobacterium paludosum* (90% of similarity) was found as dominant in glucose-fed MFC [115]. This acetogenic bacteria can ferment different sugars to acetate [116]. Found in minority as compared to the previous genus, DGGE analysis shows a band corresponding to *Clostridium* (9% in abundance). Among these genus, *C. chromiireducens* was found at ~10% in MFC against ~3% in MFC with open circuit suggesting that this bacterium grows in interaction with EABs while producing acetate. Indeed, this bacterium is able to ferment over fifteen carbohydrates to acetate but also butyrate, formate and

lactate. In the same MFC, *Enterobacter asburiae* was present at ~7.5% against 4% in open circuit. *E. asburiae* can ferment glucose and several other compounds [117].

1.3.3.2.2 Glucose to lactate

Lactic acid fermentation is a biochemical process by which glucose is converted in two steps to cellular energy and lactate [118]. The first step is pyruvate production ($\Delta G^{\circ} = -112.1 \text{ kJ mol}^{-1}$) and then its oxidation to lactate ($\Delta G^{\circ} = -43.2 \text{ kJ mol}^{-1}$) (Table 4.2). The final energy balance is $\Delta G^{\circ} = -197 \text{ kJ mol}^{-1}$. *Lactococcus raffinolactis* was found at ~7.3% in MFC and is able to ferment glucose to lactate but also two α -galactosides, raffinose and melibiose as well as lactose (β -Galactosides) and galactose, making it a fermentative bacteria of interest in the case of fermentation of complex substrates (e.g. dairy industry) [119]. In the same study, *Dysgonomonas sp.* was found at 15.6%. This genus is able to oxidize approximately fifteen carbohydrates including glucose with the production of acids but without gas [120]. However, it was isolated from human clinical specimens with a maximum growth temperature from 25°C to 37°C. Its presence in a biofilm at 4°C could be attributed to complex syntrophic interactions in this biofilm which could accelerate the metabolic rate of *Dysgonomonas* to oxidize substrates in psychrophilic conditions [110]. So, there is a difference between conventional lactic acid bacteria (*Lactobacillus*, *Streptococcus*, *Leuconostoc*) and those present in BESs.

1.3.3.2.3 Glucose to propionate

Glucose can be fermented to propionate through the intermediate pyruvate. Pyruvate conversion to propionate is thermodynamically favourable with $\Delta G^{\circ} = -123 \text{ kJ mol}^{-1}$ (Table 4.2). The overall energy balance of propionate oxidation from glucose is $\Delta G^{\circ} = -358.1 \text{ kJ mol}^{-1}$. The fermentative bacteria *Paludibacter propionicigenes* produces propionate and acetate from glucose at a ratio of 2:1 in MFC at ~6% in abundance [110]. *P. propionicigenes* produced two different metabolites and should be in interaction with EABs able to convert them in current.

1.3.3.2.4 Glucose to butyrate

Butyrate can also be produced from glucose. After producing pyruvate, it is converted to butyrate by classical routes of acetyl-S coenzyme A condensation [121]. The reaction is exergonic with $\Delta G^{\circ} = -264.8 \text{ kJ mol}^{-1}$ (Table 4.2). Two microorganisms found in MFC are able to perform this reaction: *Clostridium chromiireducens* and *Enterobacter asburiae*, present at ~10% and ~7.5% respectively in an anodic biofilm [108]. The abundance of the bacteria is 50% less in the case of an open circuit (no electron transfer through anode) which shows a possible interaction with EABs. These two bacteria can also produce acetate and would thus preferentially choose this metabolic route that favour direct syntrophy with EABs to commonly recover more energy. No EAB able to convert directly butyrate to current, suggesting that this pathway is not present in the reactor.

1.3.3.2.5 Glucose to ethanol

Glucose can also be converted to ethanol through alcoholic fermentation. In this pathway, one glucose molecule produced two pyruvates. The two molecules produced are then broken down into two acetaldehydes. Then, the two acetaldehydes are converted to two ethanol utilizing protons from NADH regenerated in NAD^+ . Glucose into ethanol is thermodynamically favourable with $\Delta G^{\circ} = -235 \text{ kJ mol}^{-1}$ (Table.2). *Escherichia coli* was detected in a MFC at ~1% in electroactive biofilm. Its low abundance appears to be due to the presence of other glucose fermenting organisms such as *Clostridium* (~18% in abundance) [112]. *E. coli* can generate ethanol with a key enzyme: the alcohol dehydrogenase. However, *E. coli* can also produce other molecules from glucose such as acetate. Its low abundance and production of many other products appears to minimize ethanol production. No known EAB able to produce electricity from ethanol was found in the biofilm. The possible way to metabolize ethanol is through formate and hydrogen which are easily converted into electricity, even though competitors such as methanogens may emerge.

At this stage, glucose is converted into various fermentation products. These products can then be converted into current by EABs or fermented again to give for example acetate and hydrogen, which may be converted to current. The next sections will describe microorganisms in glucose-fed BESs able to use products from the glucose breakdown to better understand the electroactive ecosystem functioning.

1.3.3.2.6 Pyruvate to mixed end products

Pyruvate is a platform metabolite produced at the end of glycolysis and its fermentation results in the production of mixed end products. Interestingly, a rare microorganism *Sedimentibacter hydroxybenzoicus* was found in microbial fuel cell in minority (~1%) [112]. This bacterium can live in strict anaerobic conditions and growth is supported by the fermentation of pyruvate to acetate, butyrate, isovalerate and isobutyrate with no hydrogen production [122]. Moreover, DGGE analysis showed *Desulfovibrio alcoholovorans* in MFC [115]. This strict anaerobic and sulphate-reducing organism can also use pyruvate as electron donor. However, *D. alcoholovorans* can use other fermentation end products (H₂, formate, lactate, ethanol) [123]. In view of its metabolic versatility, this bacterium could have therefore different roles in the glucose breakdown. Finally, pyruvate fermentation to propionate and acetate can be carried out by *Pelobacter propionicus*. This bacterium was detected on DGGE band in a glucose-fed MFC [67]. In this study, the main exoelectrogen was *Geobacter sulfurreducens* which cannot oxidize propionate. Therefore, it seems highly probable that, in case of syntrophic interaction, acetate is the main metabolite produced.

1.3.3.2.7 Lactate to mixed end products

Lactate issued from lactic acid fermentation may then be converted into simpler molecular product such as acetate or hydrogen. This reaction is exergonic with $\Delta G^{\circ} = -4.2 \text{ kJ mol}^{-1}$ (Table 4.2). As free energy is close to zero, the acidity and hydrogen partial pressure are two major parameters on the reaction balance. Two microorganisms capable of such reaction have already been found in bioelectrochemical systems. One is *Petrimonas sulfuriphila* able to

ferment glucose, but also lactate and more than a dozen other carbohydrate substrates [124]. The second, *Acetobacterium paludosum*, can use lactate as electron donor [116]. It is interesting to note that these two strains may be present from the beginning to the end of glucose oxidation. Indeed, they can convert glucose to acetate and hydrogen but are in competition with other glucose fermenters. Secondly, when glucose is completely consumed and intermediates are formed as lactate, they may use it as carbon and energy source. Since both products of this strain can be easily metabolized by exoelectrogens such as *Geobacter*, they can be continually in syntrophic mode in order to decrease feedback inhibition and get better metabolic performances than other fermentative bacteria. This trophic mode gives them a strong ecological advantage in electroactive biofilms. Propionate, butyrate and ethanol fermenters in BESs have not been characterized in literature. Further studies including high-throughput sequencing should be performed for better detection of these. Then, fermentation end products formed can then be converted into current as described in the next section.

1.3.3.3 Mixed end products to current

As mentioned before, the fermentation end products can be converted to current by EABs. It is interesting to compare microbial communities when single substrate is available against those presents in glucose-fed BESs. Among EABs in direct contact with anode, *Geobacter* is present in glucose-fed reactors (~15% of abundance). It is represented by these different species (*G. sulfurreducens* PCA, *G. metallireducens*, *G. pickeringii*) [12, 110]. *Geobacter pickeringii*, which was not found in BESs powered with single substrates, has been

found in MFC in presence of light. It is probably exoelectrogen and can oxidize many fermentation end products such as ethanol, lactate, or butyrate with Fe(III), S⁰ and may be electrode as electrons acceptors [125]. Its metabolic capabilities could make him an interesting exoelectrogen when several end products are simultaneously presents. Like the previous genus, *Aeromonas* can directly convert glucose to current via c-type cytochromes and hydrogen to generate power which can give a strong advantage to establish syntrophies with fermentative microorganisms [108]. c-type cytochromes are also used by *Rhodopseudomonas palustris* found at 27% on anode biofilm of ethanol-fed MFC and present at 32% with glucose, suggesting that it could have an important role as EAB such as converting acetate to electricity [92, 98]. Electron transfer via c-type cytochromes can also be performed by minority EABs as *Desulfovibrio desulfuricans*. *D. desulfuricans* was found in two glucose-fed BESs at 1% abundance [67, 110]. Its low abundance suggests that this bacterium has no significance influence on biofilm metabolism. However, this EAB is able to use lactate as electron donor [63]. This microbe was also observed in lactate-fed MFC and is interesting in the electroactive biofilms in the case of lactic acid fermentation [126]. Therefore, many EABs found in single substrate systems are also present in glucose-fed MFC. Ability to use several electron donors may explain that some EABs are present only with glucose.

Although the direct contact with anode (nanowires, c-type cytochromes) for electron transfer is present in glucose-fed BESs, EABs with lesser-known transfer mode were observed in majority in the electroactive ecosystem as *Arcobacter sp.* Members of this genus were found at 80% abundance in DGGE band in formate-fed MFC. *A. butzleri*, present in 3.2% in the anodic community of glucose-fed MFC, can convert acetate and lactate to current. Concerning electron

transfer, *A. butzleri* presents lateral appendages which could be used like nanowires [127]. Even less known, *Desulfuromonas chlorethnica* was not found in BESs powered with single substrate but with glucose. *D. chlorethnica* can use acetate as electron donor and Fe(III) as electron acceptor and could therefore, be an exoelectrogen [67].

1.3.4 Microbial communities for wastewater substrates degradation

BESs can be used in wastewater treatment to remove dissolved organic matter with low energy requirement as compared to conventional aerobic treatment. MFCs yield 50-90% less solids to be disposed off, reducing restatements costs [128]. BESs are more appropriate than anaerobic digestion to treat low-strength wastewater such as municipal wastewater. In addition, BESs allow direct harvesting of electricity in contrast to anaerobic digestion which requires a two-step process: methane generation followed by cogeneration in an internal combustion engine [129]. The multitude of organic compounds present in wastewaters can support BESs operation. Hydrogen (MEC) or electricity (MFC) can be produced from the fermentation of the organic matter contained in wastewaters. COD removal can reach up to 95% with double cell MFC which is competitive process as compared to activated sludge [130]. The wastewater compositions are very different, depending on their origins (industry or domestic). For example, wastewaters from grain industry have a high content of ethanol (221 mg L^{-1}) as compared to that of lagoon (33.6 mg L^{-1}) while the latter has high propionate content (245 mg L^{-1}) as compared to the grain industry (10.6 mg L^{-1}) [130]. Microbial ecosystems in wastewater-fed BESs are very complex. Two main steps to convert wastewater substrates into energy are

fermentation that will produce low molecular weight compounds (volatile fatty acids, alcohols) and their conversion into current by EABs.

Microorganisms that ferment wastewater substrates are very diverse in view of the diversity of compounds to be oxidized. *Parabacteroides* has been found in 4.7% in wastewater-fed MEC. A strain isolated from human faeces, can oxidize many carbohydrates (L-arabinose, L-rhamnose, Glucose, Lactose, Maltose, D-mannose, D-raffinose, Sucrose, D-trehalose, D-xylose) to form acetate or lactate [131]. This wide range of carbohydrate substrates is an ecological benefit in this ecosystem. In the same biofilm *Pelobacter sp.* was detected at 4.7% of abundance. As described above, this microorganism can ferment alcohols to propionate and acetate. So, the electroactive ecosystem fed with many substrates has two main fermenters, each specialized in a range of molecules. This distribution could decrease the inter-species competition, thus reducing energy lost in the competitive process (synthesis of inhibitor molecules) and stabilizing the ecosystem in long term. The common point between these two microorganisms is the molecules production like acetate or propionate. Concerning EABs, this biofilm is composed of 67% of *Geobacter* with *G. metallireducens* (23%), *G. uraniireducens* (16%), *G. sulfurreducens* (14%) and *G. lovleyi* (14%). The only known exoelectrogen that does not belong to the *Geobacter* genus found in this biofilm at 4.7% in abundance, *Alkaliphilus*, uses the long range transfer mode with flavins and can use acetate and lactate as electron donor [132]. The exoelectrogens represent more than two thirds of the biofilm, which prevents fermenters against feedback inhibition by reloading NAD^+ , essential for the fermentation process and high COD removal. [7, 133]. It is interesting to note that EABs present in BESs fueled with single substrates, such as *Geobacter sp.*, are also present and dominant with

wastewater. This could be due to two reasons: (1) there is a strong selection pressure by anodic material. Microorganisms able to do extracellular electron transfer to the anode as final electron acceptor. EABs will have a great energetic advantage against other bacteria. (2) Fermentation end products from wastewaters are mainly acetate and propionate, which also restricts the number of EABs able to convert it into current, this favors specific EABs genus and could decrease the bacterial diversity.

Conclusion

Electroactive biofilms are complex structures capable of converting organic matter into electric current. This is possible through the establishment of syntrophic interactions among the microorganisms that oxidize organic matter to various end-products, and EABs that convert these simple molecules into current. The microbe-electrode exchange is the result of an evolutionary process developed over millions of years, resulting in the development of different electron transfer modes. Among the many EABs, *Geobacter* is the most studied. It is also often the most abundant in the anodic community and has the ability to develop nanowires allowing direct-interspecies electron transfer, a new mode of interaction. Degradation pathways of fermentation end products are specific to each substrate, with a common final point, acetate and hydrogen; both products enable a rapid transfer through EABs. The main concurrent organisms are methanogens, able to use efficiently hydrogen as compared to electroactive bacteria, causing a decrease of the coulombic efficiency. Glucose fermentation produces many compounds, each with its own oxidation pathway. Many microorganisms are commonly

present with glucose, including specific EABs developing syntrophy with fermentative organisms. Finally, wastewaters which have a wide range of organic substrates can be effectively used in BESs. The omnipresence of *Geobacter* shows how this genus is important at both small and large scale. However, there is still a large unexplained part with regard to the role of microorganisms in the electroactive biofilm.

To improve stability, performance and coulombic efficiency, it is necessary to better understand the ecology of electroactive biofilms, the different interaction modes (direct, indirect) according to the substrate and use this knowledge to promote the EAB-fermenter interaction to prevent methanogenesis and enable this technology to emerge in the future.

1.4 Substrate-based pre-acclimation strategies: eco-engineering solution to improve MXCs performance

Introduction

The structure of microbial communities growing on MXCs bio-anodes are highly affected by the type of electron donor in the medium [7]. Currently, many substrates have been used for the study of electroactive biofilms such as acetate, lactate, butyrate or propionate [12]. But among them, acetate is the most studied metabolite. The rapid growth of biofilm on the anode is favored by acetate which is easily used by electroactive bacteria [66]. Of these, *Geobacter sulfurreducens* was found in the majority of cases predominantly on the anode [11]. Indeed, it develops rapidly and colonizes the only available electron acceptor at the expense of

other taxa [62, 92, 134-136]. Such preponderance minimizes, if not excludes, the presence of other bacteria that can provide complementary ecological functions such as fermenter [137]. *G. sulfurreducens* has a very low metabolic versatility making it ineffective for oxidizing molecules other than acetate, lactate or hydrogen [60]. Therefore, when acetate is available in solution, *Geobacter sulfurreducens* is predominant, which will directly impact the conversion of other molecules such as propionate or butyrate to electrons, lowering the performance of MXCs in terms current density [138].

To avoid the predominance of this electroactive bacteria and thus the associated barrier effect, it is possible to use eco-engineering techniques. The latter aim to modify the microbial structure of the biofilm in a controlled way in order to redirect it to defined functions and/or desired performances. In MXCs, the main performances indices are the current density per surface area ($A. m^{-2}$) or per volume ($A. m^{-3}$), the electron consumption over a given time period (or COD removal), the coulombic efficiency (%) and the start-up time. It is therefore possible, in ecological studies, to link a microbial consortium with these different indices (by regression techniques for example) in order to identify effective or ineffective bacteria for the conversion of substrates into current.

Many eco-engineering techniques have been described in MXCs such as the modification of anodic potential (MECs), external resistance (MFCs), the addition of electronic mediators (e.g. humic substances, flavins) or substrate-based pre-acclimation strategies [136, 139-144]. Among these methods, substrate-based pre-acclimation appears to be the most

attractive because many studies have demonstrated the relationship between substrate and communities structures [7]. The main goal of this method is not only the improvement of MXC performance indices but also an increase of the capacity of adaptation to different substrates (i.e. improve resilience to substrate variations). Microbiological analysis by NGS makes now possible to compare the performances of the MXCs in relation with the consortia present, according to the type of pre-acclimations. This method is generic as it can be applied for both MECs and MFCs. Fig. 1-8 summarizes the eco-engineering processes used in MXCs. Substrate-based pre-acclimation studies are categorized into two groups: the first deals with synthetic media and the addition of defined substrates; the second category corresponds to the use of real effluents with either pre-acclimation with defined substrates or wastewaters. The next part will describe the pre-acclimation strategies in synthetic media.

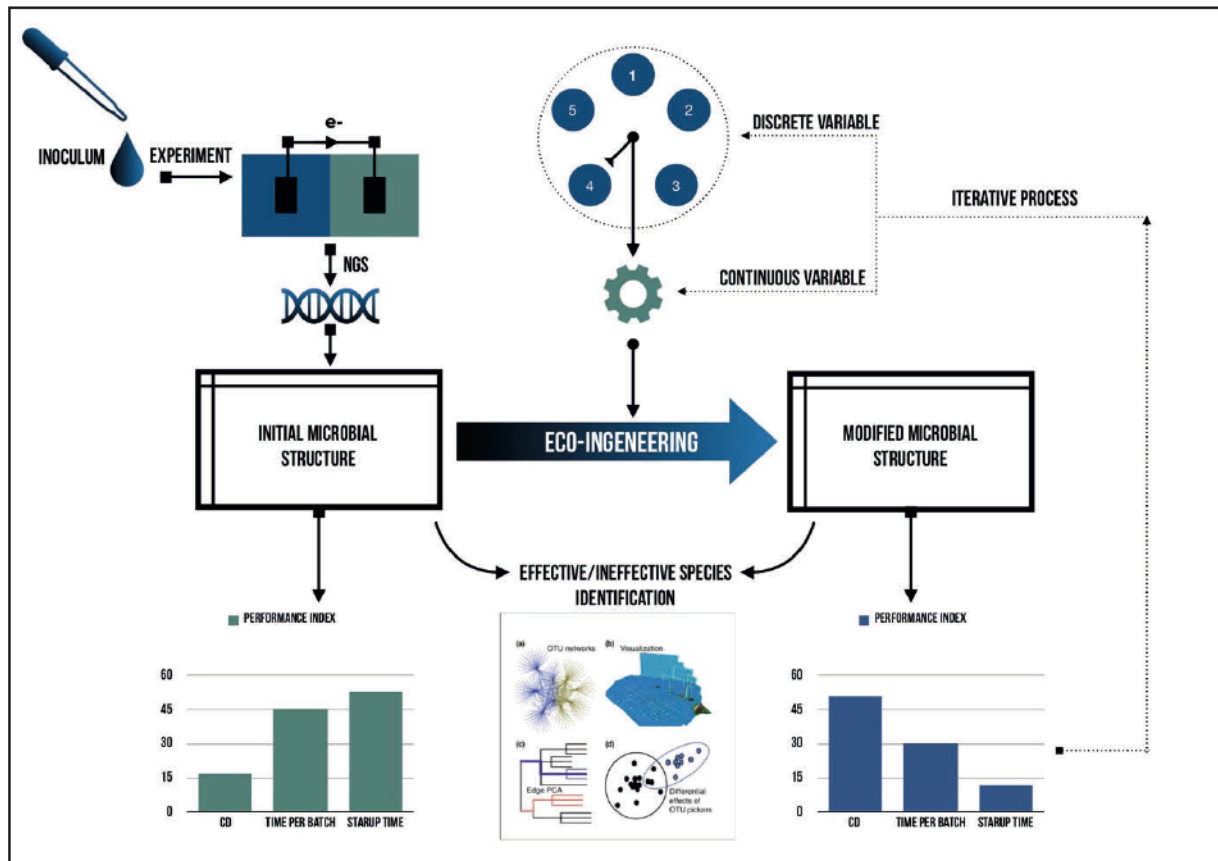


Fig. 1-8 Overall view of the Eco-engineering techniques application to bioelectrochemical systems in order to improve the desired performance by modifying the structure of the microbial ecosystem (planktonic and/or anodic). Discrete variables can be: (1) E(anode) (V) or external resistance (Ω), (2) Electronic shuttles. (3) Anodic material. (4) Substrate, (5) Others (Mixed inocula; Inoculum dilution series; Use of quorum sensing auto-inducers; enrichment of electroactive bacteria by Fe (III)). Continuous variables can be: concentration variation (Substrate, Electronic shuttles, Inoculum), pore size (anodic materials).

The different studies presented in the following sections do not use a similar vocabulary, probably because it is a recent disciplinary field (first study in 2008). For a better understanding, four concepts have been considered: (1) the “switch strategy” is when an MXC is used with two different simple substrates (or multi-substrates) or wastewaters, one after the other (e.g. during two consecutive batches) (Fig. 1-9. A); (2) the “mix strategy” is when a simple substrate

(or multi-substrates) or wastewater is added in mixture with either another wastewater or a simple substrate (or multi-substrates) (Fig. 1-9. B); (3) 'no-acclimated control', which defines a condition under which no prior acclimation has been set up (e.g. a single batch) (Fig. 1-9. D); (4) switchless- or unmixed-control defines a condition where no substrate change has been carried out from one batch to another (pre-acclimated anode with final substrate) (Fig. 1-9. C).

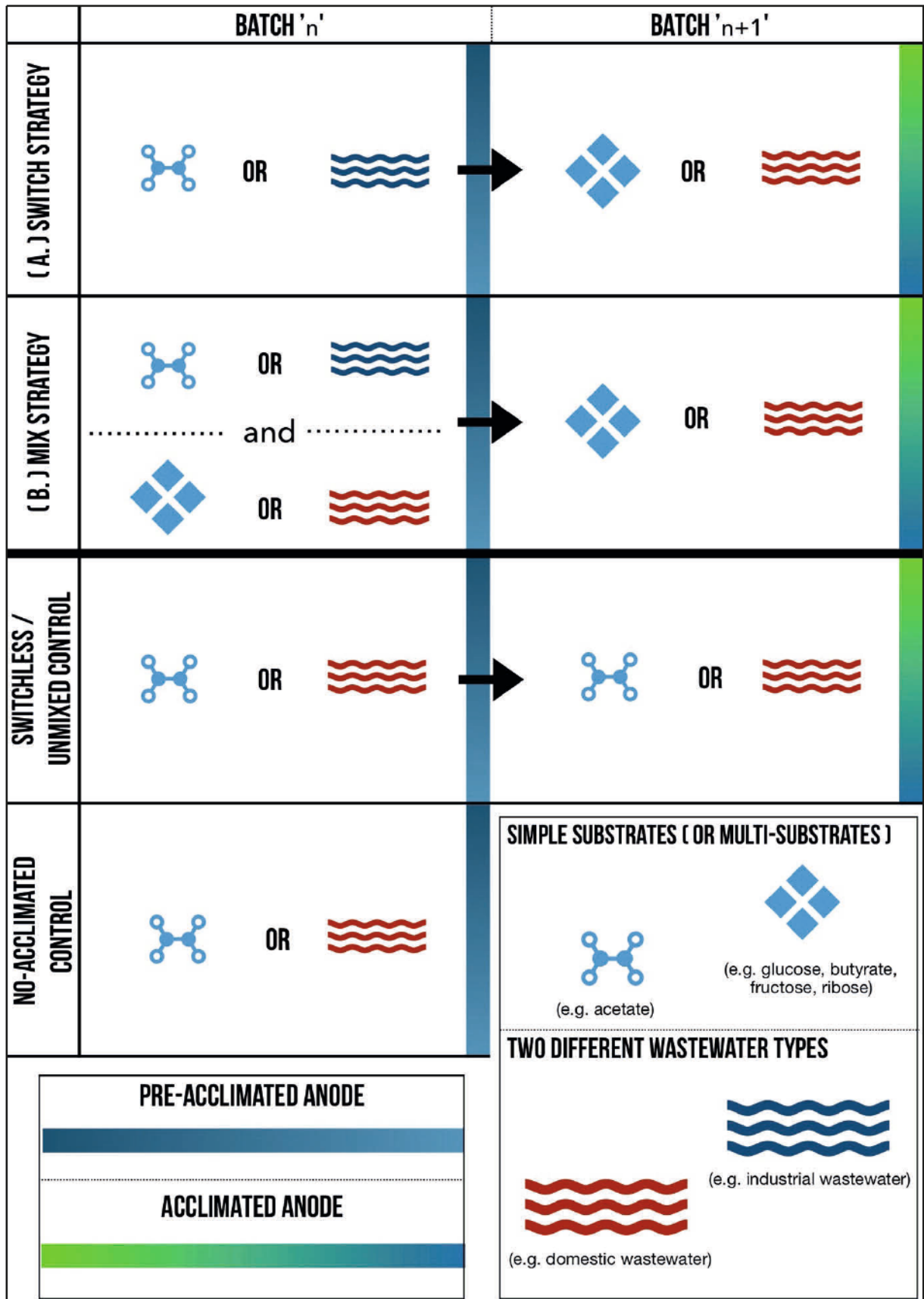


Fig. 1-9 Summary of the different typologies used for the description of substrates based pre-acclimated MXCs. The possibilities illustrated are given as examples.

1.4.1 Synthetic medium

Synthetic media were used first to study the impact of a substrate change (cross-feeding) after a pre-acclimation period. The advantage of controlling all physio-chemical parameters to link performance improvements and the associated microbial ecosystem is enabled by synthetic medium.

1.4.1.1 *Pre-acclimation studies: initial studies*

The two pioneer studies in this domain used pre-acclimation techniques without changing substrate. The following two studies are not in the proposed classification (Fig. 1-9), but introduce the interest of pre-acclimated microbial consortia. Liu et al. (2008) propose a pre-acclimation strategy with a synthetic medium under a fed-batch MFC [145]. This study examines a new three-step strategy: (1) a primary biofilm was formed with acetate (10 mM) and an inoculum (from wastewater) for 850 hours; (2) the anode was transferred to a new reactor containing a novel anode and acetate (10 mM) without external inoculation (the primary biofilm was used as inoculum) for 150 hours; (3) on the new anode, a secondary biofilm developed (Fig. 1-10). The latter produces twice as much current with a decrease of the start-up time from 150 to 20 hours. Here, the authors present a simple and effective way to improve the conversion

of acetate to current. The primary anode being enriched with EABs capable of using acetate is therefore a suitable inoculum for the desired biochemical reaction.

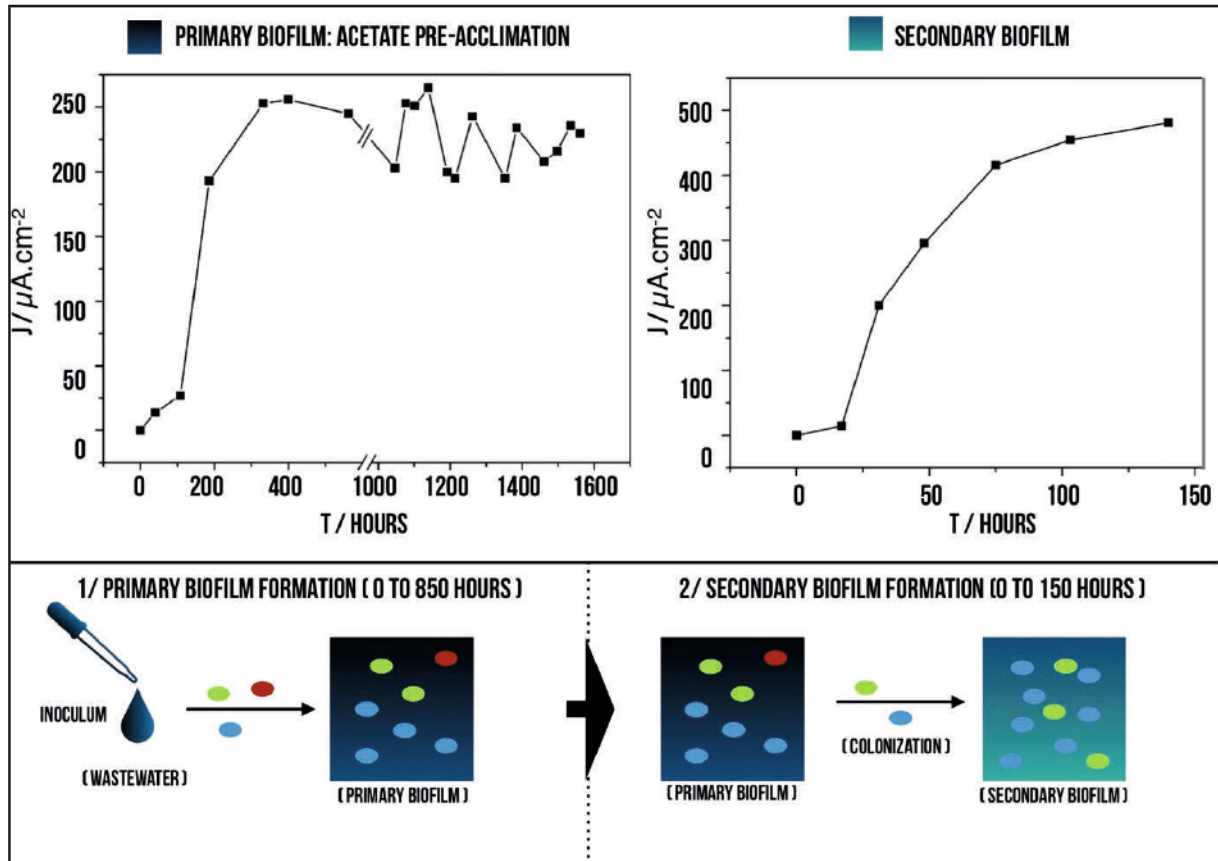


Fig. 1-10 Maximum current densities of primary (left) and secondary (right) biofilms. The discontinuity observed between 850 and 1000 hours (for primary biofilm) corresponds to the time of transfer for secondary biofilm formation (Adapted from Liu et al. 2008).

In the second study, the same substrate was used to acclimate an anode in fed-batch mode MEC with the aim of improving the conversion of glycerol into current and increase hydrogen production [146]. Acetate was periodically added at increasing loads during 18 days to pre-acclimate the electroactive communities to a high concentration of this substrate, one of

glycerol fermentation product. With this study, the authors introduced a successful substrate-based strategy (Fig. 1-11).

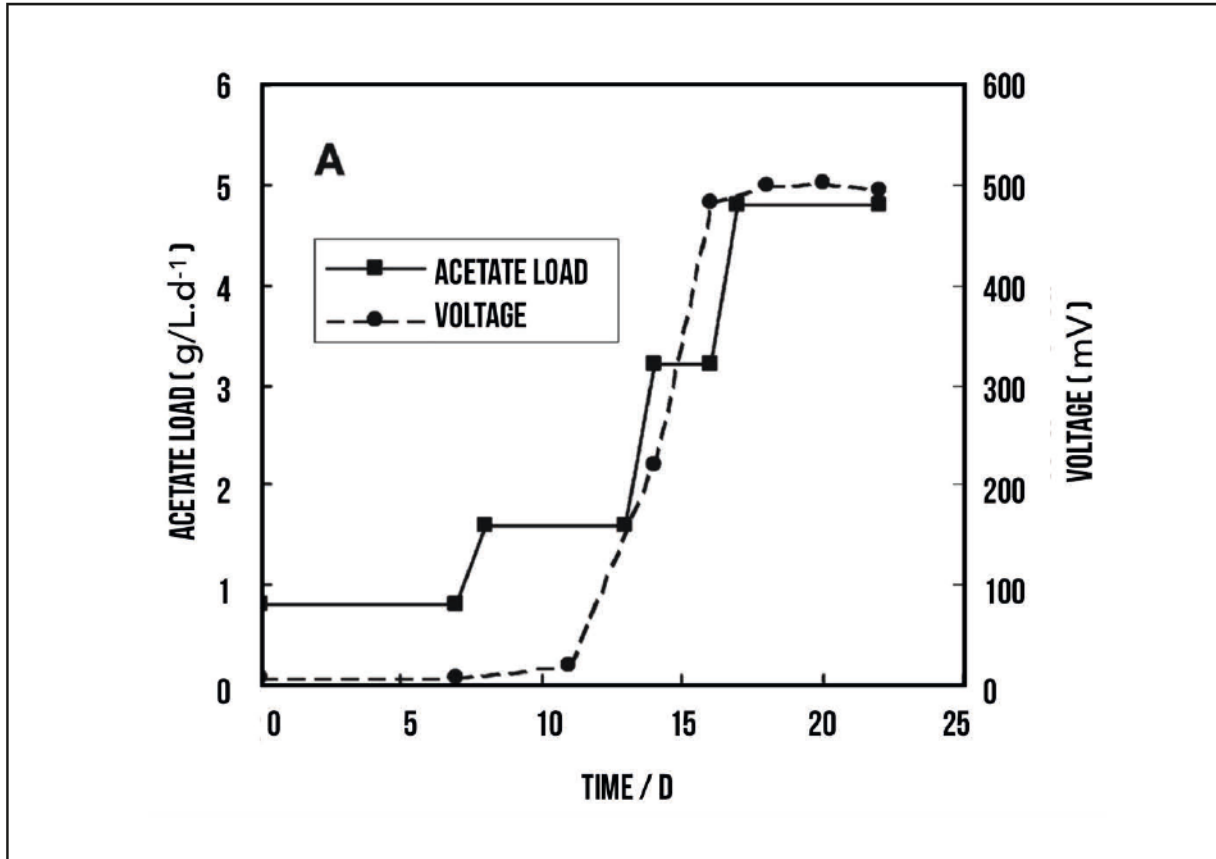


Fig. 1-11 Increase of acetate load during the start-up process in MFC mode. (Adapted from Escapa et al. 2009)

1.4.1.2 Switch strategy

The switch strategy consists in using two different substrates, one after the other (e.g. during two consecutive batches) in MXCs (Fig. 1-9. A). If the same substrate is used during all the experiment, the condition is called “switchless-control”, necessary to compare performance indices (Fig. 1-9. C). To better understand the impact of a substrate switch on

performances and microbial ecosystems, Zhang et al., (2011) investigated MFC bio-anodes pre-acclimation with three different simple substrates: acetate, glucose and butyrate in fed-batch mode [147]. Then, during cross-feeding, each single substrate was exchanged with another as shown in Fig. 1-12. The MFCs pre-acclimated with glucose or butyrate had a higher voltage after cross-feeding compared to those pre-acclimated with acetate, with 550, 520 versus 480 mV respectively. Planktonic and anodic microbial community analyses were performed before and after the substrate switch from acetate to glucose, revealing the growth of taxa such as *Clostridium* and *Bacilli*, able to ferment many carbohydrates such as glucose. On the contrary, the switch from glucose to acetate caused the emergence of the *Geobacter* genus whereas the *Firmicutes* completely disappeared. This seems consistent with the fact that acetate can be easily oxidized at the anode by bacteria of the *Geobacter* genus without the need for fermentative bacteria. As measured, acetate was the main fermentative product of glucose fermentation (~8 mM) next to ~1 mM for propionate and butyrate. Consequently, the glucose-acclimated ecosystem is able to use acetate, which could explain the very fast adaptation of this microbial consortium to the substrate switch.

Therefore, this study shows that glucose pre-acclimation is a preferable way because the electroactive ecosystem is able to acclimatize more rapidly after cross-feeding with acetate and produce a higher voltage compared to acetate before cross-feeding with glucose.

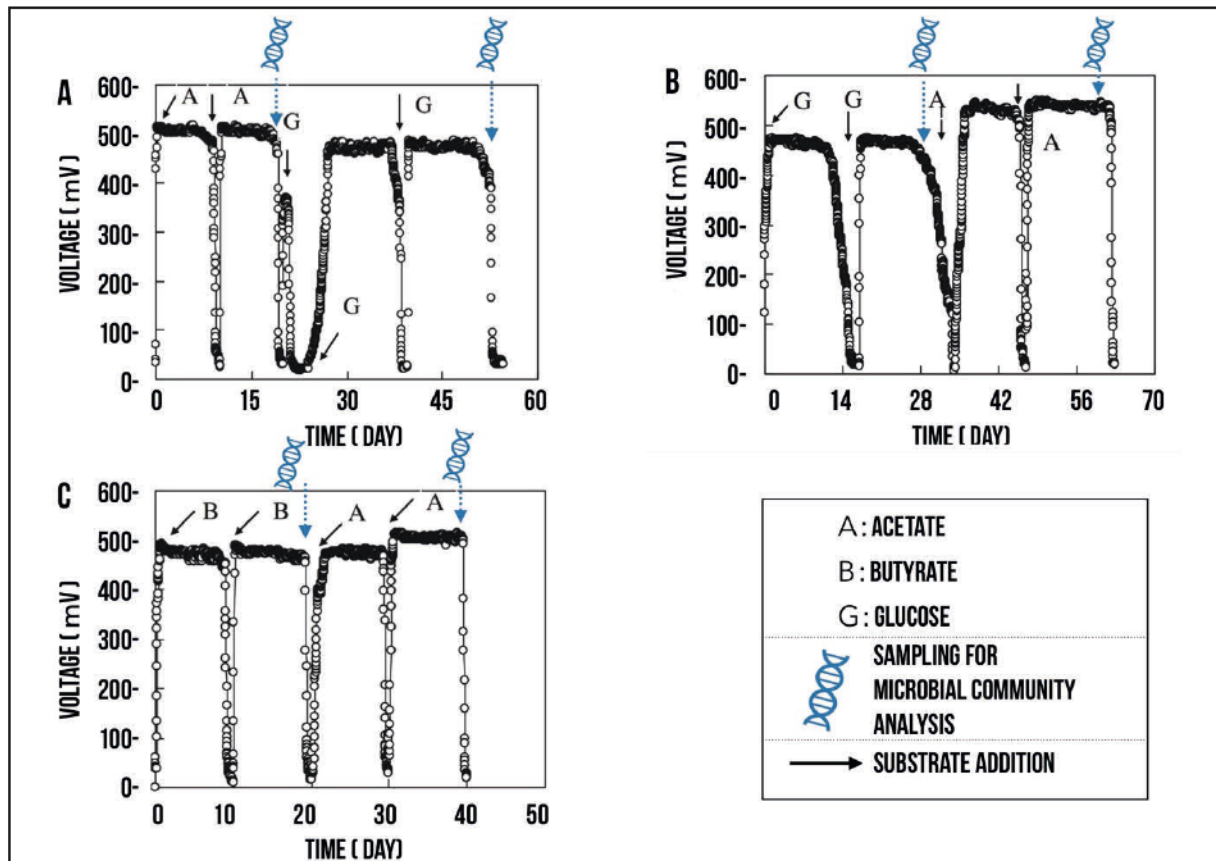


Fig. 1-12 Effect of substrate switching on voltage generation. (a) Glucose was added into acetate-enriched MFCs; (b) Acetate was added into glucose-enriched MFCs; (c) Acetate was added into butyrate-enriched MFCs. The concentration for each substrate was 1000 mg-COD/L. (Adapted from Zang et al. 2011).

Glucose was also used to study the impact of microbial pre-acclimation on hydrogen production in fed-batch mode MECs using two glucose fermentation products: acetate and butyrate [93]. These two electron donors were first used separately, then in a mixture (50% acetate; 50% butyrate) and finally switched from one to the other. The current densities were identical during pre-acclimation between the acetate- and butyrate-fed MECs. After the switch, butyrate pre-acclimation CD increased from 1.2 to 1.8 A.m⁻² in butyrate pre-acclimated MEC fed with acetate as substrate, meanwhile the acetate pre-acclimated MEC decrease to 0.2 A.m⁻²

² when fed with butyrate (Fig. 1-13). In addition, butyrate pre-acclimated MEC was the most efficient process with a 25% increase in hydrogen production while the acetate pre-acclimated MEC was not able to produce current when fed with butyrate alone (Fig. 1-13). Moreover, pre-acclimation with butyrate allowed a 58% decrease of methane production in the anode. This decrease in methane production is interesting because methanogenesis is the main cause of coulombic efficiency decrease in MXC [65].

To better understand the ecological mechanisms involved, 16S rRNA sequencing was also carried out. In this case, two different communities were observed with, on one side, the acetate-acclimated bio-anode dominated by the *Geobacter* (EAB) and *Spirochaetes* genera (known for homoacetogenesis from H₂ and CO₂). On the other side, the butyrate-acclimated bio-anode was dominated by the *Synergistaceae* and *Syntrophomonadaceae* families [148, 149]. The development of a consortium with low metabolic versatility is favored by acetate and ineffective during a substrate change contrary to a butyrate pre-acclimation, which promoted the growth of a syntrophic consortium having a higher metabolic versatility and leading to an increase in current density.

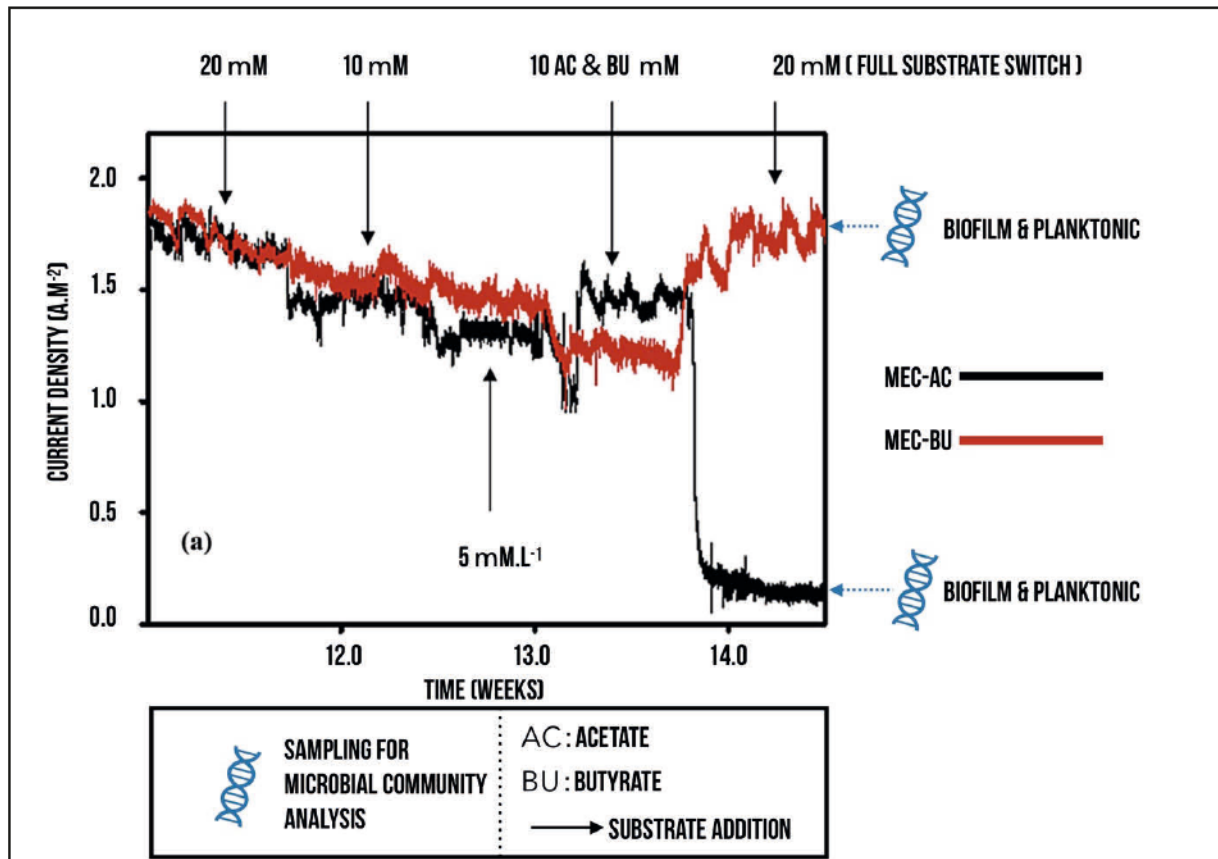


Fig. 1-13. Variation in the current densities over time, for acetate (MEC reactor AC) and butyrate (MEC reactor BU) (From Popov et al. 2016).

In a study conducted in fed-batch MFCs, acetate pre-acclimation showed the lowest performances in COD removal when the MFCs were fed with butyrate or sucrose [150]. Michie et al, (2013) tested separately three different electron donors, i.e acetate, butyrate and sucrose, for pre-acclimation in fed-batch mode as well as switchless-control (same substrate over time). The MFCs were fed on a weekly basis and operated over a period of 83 weeks. After this acclimation period, the substrates were switched between them in all possible combinations for 50-hours (Fig. 1-14). The MECs acclimated either to acetate or butyrate were close in terms of COD removal (57.1 and 54.1 respectively) with sucrose as substrate. Interestingly, the sucrose-

acclimated MECs performed better compared to the acetate-acclimated MECs after having switch to butyrate, with 58.2 and 41.6% COD removal respectively. According to the authors, these results can be explained by a lack of fermentative bacteria in acetate pre-acclimation condition, while the other two metabolites are fermentable, which promoted the establishment of a syntrophy-based ecosystem. Sucrose is a dimer of glucose and fructose, its fermentation, depending on the metabolic pathway, produces many different molecules such as lactate, succinate, ethanol or butyrate [151]. To understand how this metabolite makes the ecosystem more efficient for the use of butyrate, 16S rRNA sequencing (biofilms & planktonic part) would be interesting with at least triplicates to identify the fermentative and electroactive bacteria involved in the conversion of sucrose and butyrate into current.

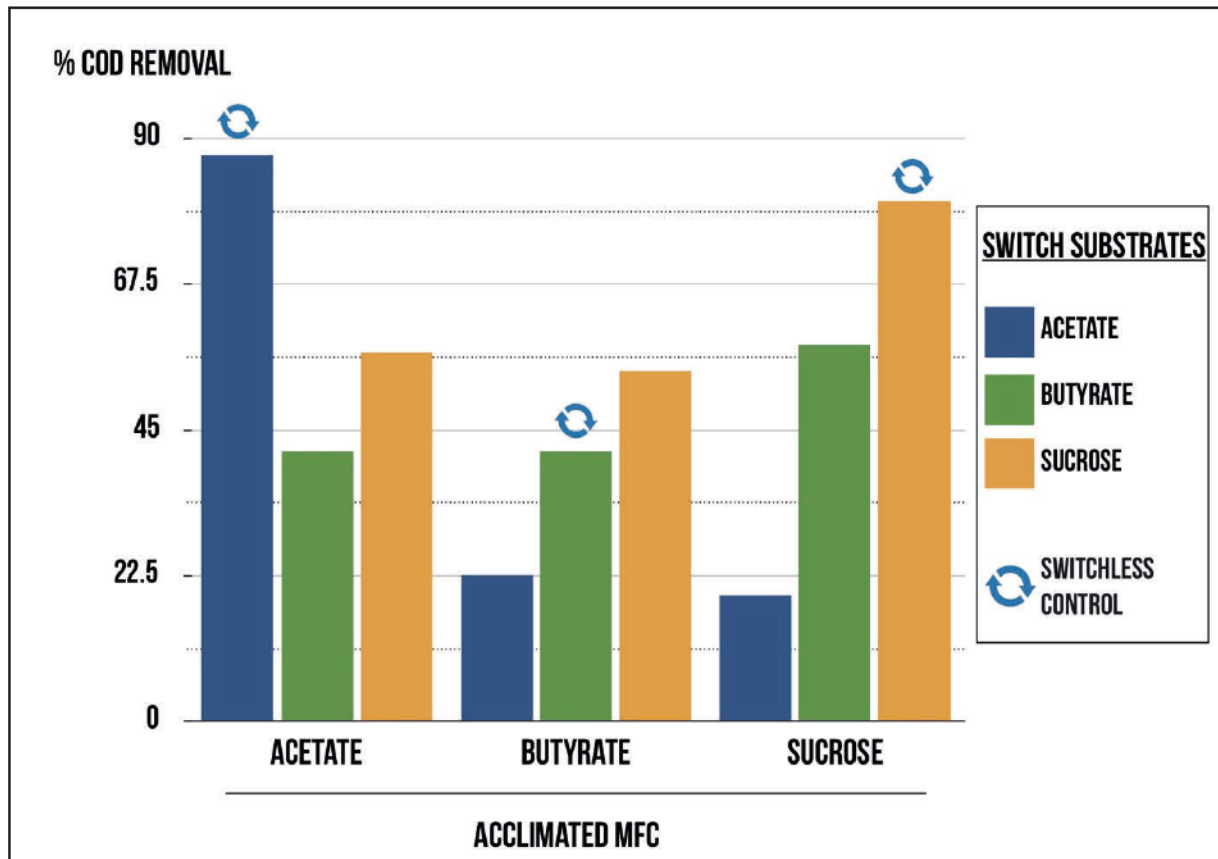


Fig. 1-14. Performance in terms of COD removal of pre-acclimated MFCs (Data from Michie et al. 2013).

Overall, the acetate-acclimated bio-anodes contained EABs specialized in the conversion of this substrate into current, resulting in ecosystems with a low metabolic versatility. In contrast, butyrate-acclimatized bio-anodes led to the development of a microbial community mainly composed of syntrophic species such as *Syntrophomonadaceae* and *Synergistaceae*, more suited to metabolic changes because these microbial families are capable of using many substrates (carboxylic acids) which can result in an ecosystem with a high metabolic versatility. The consortium would be more resilient and better adapt to cross-feeding. Nevertheless, very few studies have been carried out in that sense with few replicates, which

makes the relationship between pre-acclimation with a single substrate, the impact on the microbial ecosystem and its connection to macroscopic performances still little known. A summary of these different ecological hypotheses is shown in Fig. 1-15. The main characteristics of these studies are summarized in Table 1-3.

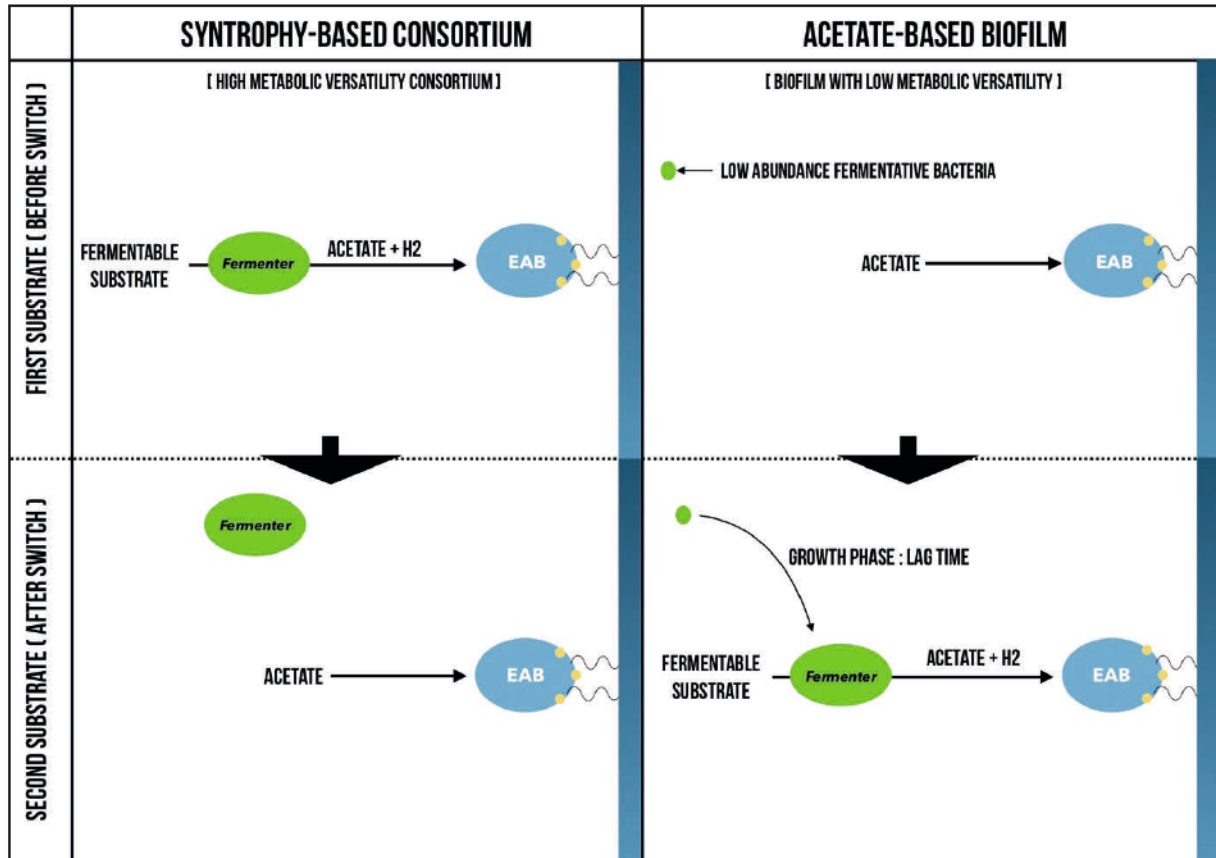


Fig. 1-15 Hypothetical interaction networks between a syntrophy-based consortium and a biofilm specialized in acetate oxidation. The electroactive bacterium is specialized in the acetate oxidation (e.g. *Geobacter sulfurreducens*). The fermentative bacterium is capable of oxidizing fermentable substrates such as carboxylic acids in syntrophic interaction (e.g. *Syntrophomonadaceae* & *Synergistaceae*).

Table 1-3 Summary of the different synthetic medium substrate based pre-acclimation studies in MXCs. Controls: switchless-, unmixed- or no-acclimation-controls

MXCs	First substrate	Switch substrates	Microbial analysis	Controls	Ref.
MFC	acetate	acetate	Scanning electron micrograph	-	[145]
MEC	acetate	glycerol	-	-	[146]
MFC	acetate; glucose; butyrate	glucose; acetate	DGGE	-	[147]
MEC	acetate; butyrate	acetate; butyrate	DGGE	-	[93]
MFC	acetate; butyrate; sucrose	acetate; butyrate; sucrose	-	switchless	[150]

1.4.2 Real wastewaters treatment

1.4.2.1 Switch strategy

Pre-acclimation methods have been further studied with organic substrates such as domestic or industrial wastewaters with the switch strategy (Fig. 1-9. A) (Table 1-4). Using wastewaters increases the number of available substrates (VFAs, proteins, amino acids, carbohydrates) which makes elucidation of the trophic network more complex compared to defined media [3]. The use of MXCs in real conditions with a perspective of a successful scale-up is favored by the use of wastewaters in pre-acclimation.

Antonopoulou et al., (2010) used the substrate-based pre-acclimation procedure for cheese whey treatment [152]. Initially, MFC was supplied with glucose (for 1700 hours), lactose (from 1700 to 2000 hours) and then cheese whey in fed-batch mode. Glucose was used to enrich a biofilm in fermentative bacteria and lactose was used because it is mainly present in cheese whey effluent (~62%). Through the glucose acclimation step, the voltage gradually increased to 45mV and then stabilized around 40 and 45 mV with lactose. After cross-feeding with cheese whey, stable voltage over time was observed (~45 mV), but CE was very low (1.9%) suggesting an electron leakage to other acceptors than the anode. In this study, it would be interesting to perform non-acclimated and switchless-controls to verify the effectiveness of this strategy (Fig. 7).

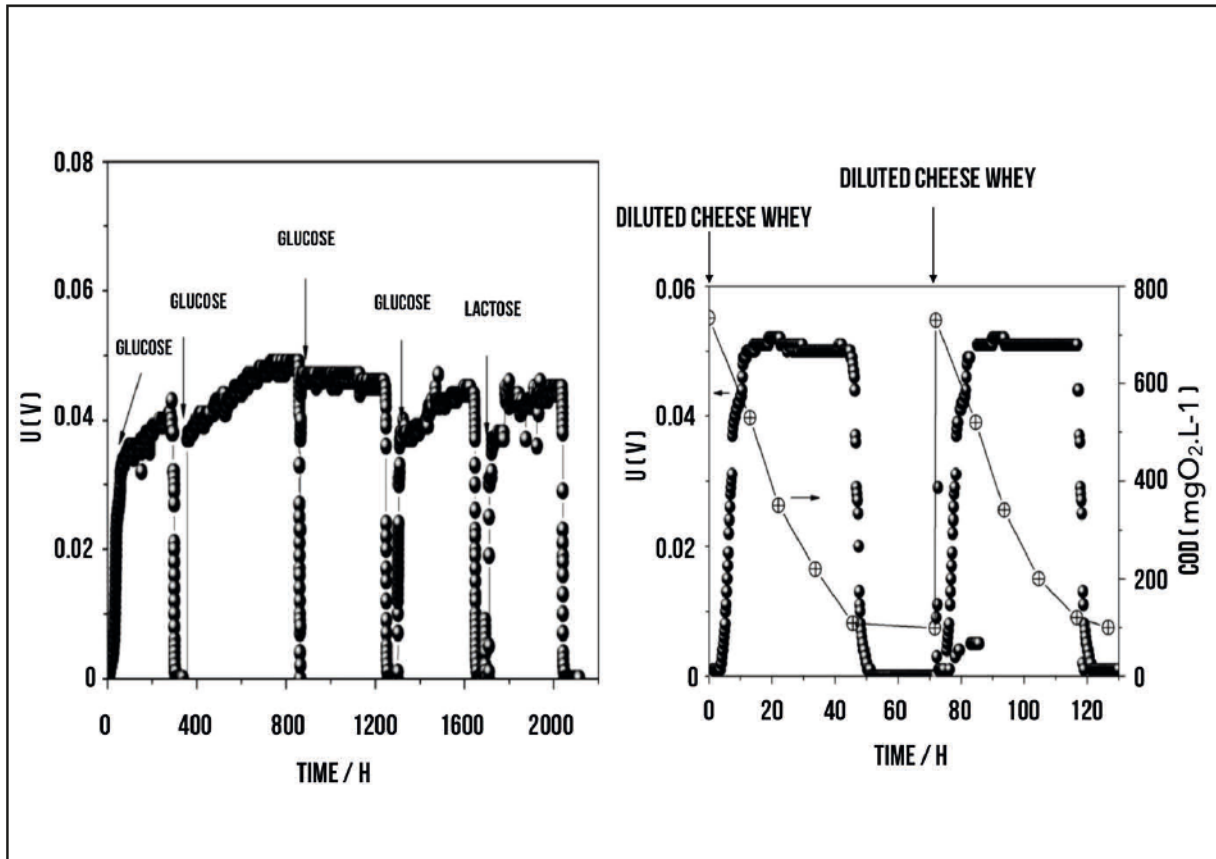


Fig. 1-16. MFC voltage vs. time when using glucose, lactose and diluted cheese whey as energy source. External load: $R=100\Omega$. (Adapted from Antonopoulou et al. 2010).

In another study, pre-acclimation with domestic wastewaters was shown to be effective for the treatment of an de-oiled refinery wastewater effluent (DOW) in fed-batch MFC [153]. The MECs were previously acclimated either with domestic wastewater (DW) or a mixture of refinery wastewater and domestic wastewater (RW + DW) or only refinery waste water (RW). After cross-feeding, the MECs were then fed with either a de-oiled refinery wastewater in triplicate (DOW 1-3) or a de-oiled oily sewer (DOSW 1).

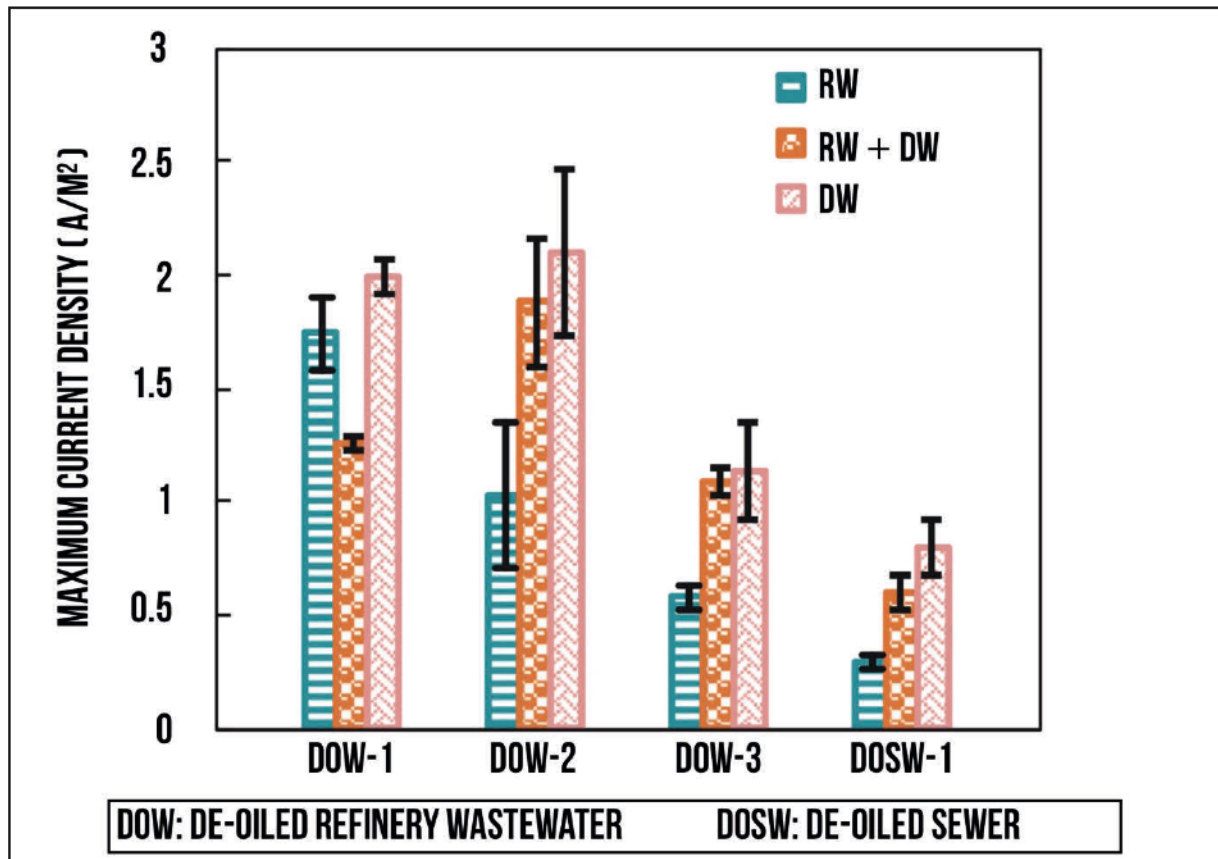


Fig. 1-17 Maximum current densities produced by the refinery wastewater samples during cross-feeding tests. MEC reactors acclimated to only refinery wastewater was indicated by “RW”, to the 50:50 refinery wastewater + domestic wastewater mixture by “RW + DW”, and to only domestic wastewater by “DW”. DOW1-3 are samples from de-oiled refinery wastewater with the associated replicate number. DOSW is a sample of de-oiled oily sewer. (Adapted from Ren et al. 2013).

These authors observed a significant improvement of current density for conditions DOW 2 & DOW 3 by pre-acclimation with domestic wastewater (DW) or in mixture (RW+DW) compared to refinery wastewater (RW) (Fig. 1-17). The enrichment of effective electroactive species at the anode was favored by the use of DW which could provide not only a diversity of substrates, but also nutrients and higher microbial diversity. Unfortunately, the authors did not provide any information about the microbial community compositions. In that

case, it would be interesting to analyze the microbial communities by NGS to see if the high performances were correlated to the development of different microbial communities.

However, it seems that domestic wastewater acclimation with effluents is not always effective. Ullery and Logan (2015) tested two pre-acclimation strategies to treat a cellulose fermentation effluent in a fed-batch MEC [4]. The first strategy consists in domestic wastewater acclimation and the second acetate acclimation. A switchless-control was performed. None of the tested strategies showed improvement in COD removal, current density or coulombic efficiency indices after cross-feeding with domestic wastewater. So, it appears necessary to develop a suitable acclimation technique, for example in the form of a screening with many possibilities (e.g. single substrates, wastewater, substrate mixtures), for each kind of wastewater to be treated.

For treating brewery wastewaters, Yu et al., (2015) compared five different substrate pre-acclimation: glucose, acetate, butyrate, propionate and a mixture of the 4 were tested in a fed-batch MFC. [154]. After cross-feeding with the brewery wastewaters, the power density produced with glucose pre-acclimation was the most efficient, whereas the propionate pre-acclimation was less efficient, with $890 \pm 12 \text{ mW.m}^{-2}$ and $297 \pm 23 \text{ mW.m}^{-2}$ respectively. In this study, microbial community DGGE analysis was carried out. Two clusters can be distinguished in the PCA analysis (Fig. 1-18). The first is composed of all pre-acclimated samples (Cluster I) and the second of all samples after the switch with the brewery wastewater (Cluster II). In DGGE analysis, differences in band intensities were observed between before

and after the switch, suggesting a high intra-cluster variability. These results suggest that the community structures were substrate-dependent because of the switch (Cluster I to Cluster II). Consortia converged to the same group, with similar microbial structures, whatever the pre-acclimation substrate. In this case, it seems complicated to relate community structure to performances and this also raises the question of the durability of the acclimation process.

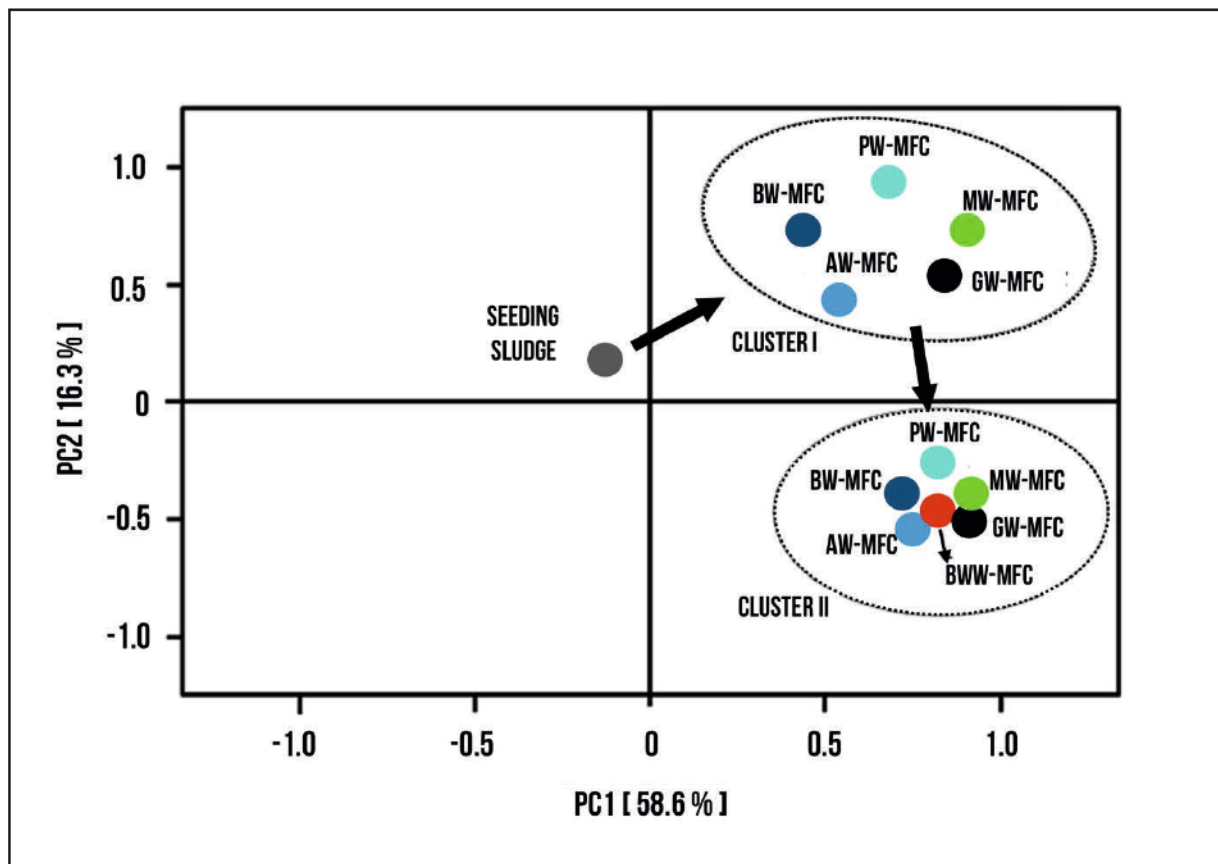


Fig. 1-18 Principal components analysis (PCA) based on the DGGE band positions and intensities in the MFCs acclimated at Cluster I (Pre-acclimation) and Cluster II (after cross feeding with brewery wastewater). BW: Butyrate-fed. PW: Propionate-fed. MW: Mixed substrates-fed. GW: Glucose-fed. AW: Acetate-fed. BWW: Brewery Wastewater (From Yu et al. 2015).

In another study, butyrate also showed its effectiveness as a pre-acclimation substrate. The latter was compared with acetate and a switchless-control on a corn stalk fermentation effluent (CSFE) in fed-batch MEC [155]. In this case, after cross-feeding with CSFE, the butyrate condition was always better than acetate- and the switchless-control with 5.21 ± 0.24 , 4.22 ± 0.19 and 4.55 ± 0.14 $\text{H}_2 \cdot \text{L}^{-1}$ CSFE respectively. Butyrate-pre-acclimation also improved the current density to 480 ± 11 , 391 ± 9 and 346 ± 11 $\text{A} \cdot \text{m}^{-3}$ for butyrate-, switchless-control- and acetate-acclimation respectively (Fig. 1-19). Interestingly, acetate condition had the worst performances for both indices. These results demonstrated that the butyrate pre-acclimated biofilm has significant advantages in CSFE treatment and could improve the hydrogen performance production in MEC. However, an NGS community analysis would be interesting to understand why the acetate-acclimated consortium was less efficient (e.g. through a lack of fermentative bacteria).

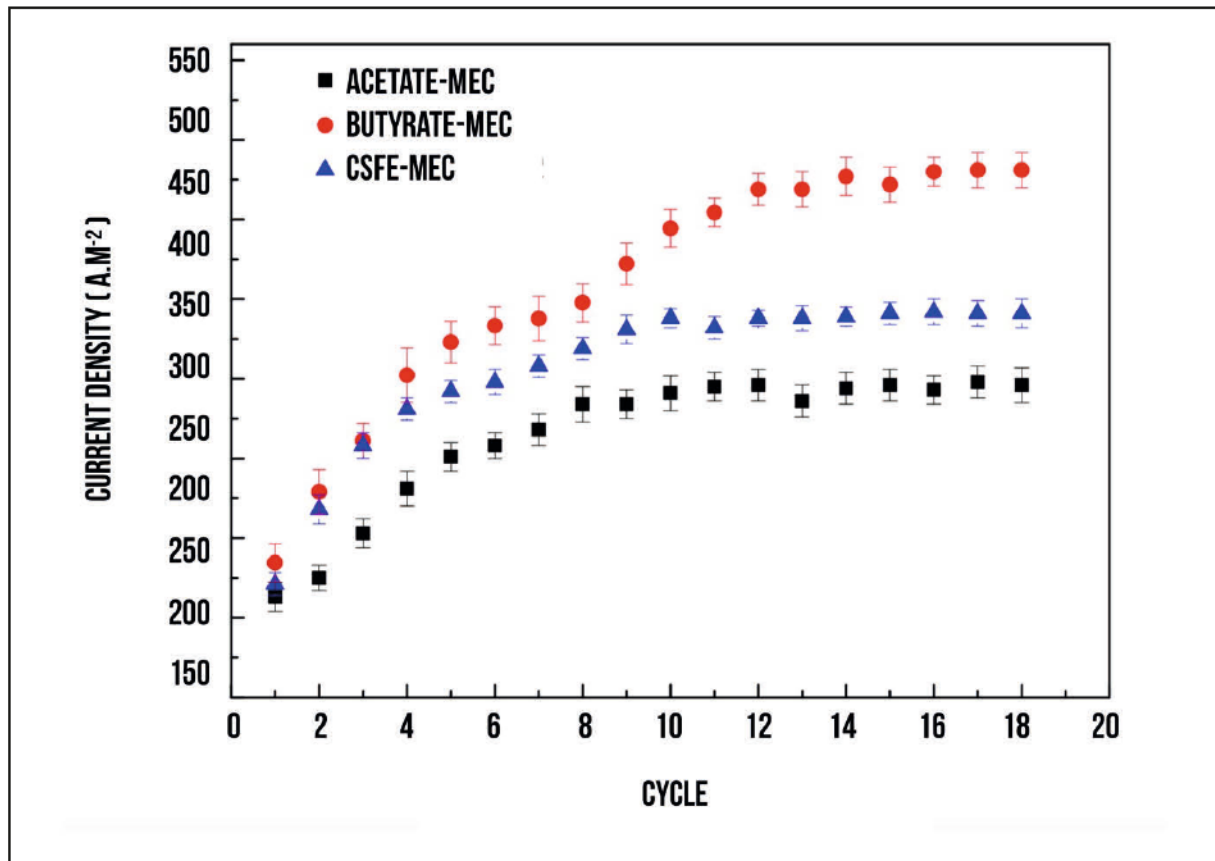


Fig. 1-19 The performance of current density during MECs start-up period. Acetate-MEC: pre-acclimated to acetate; Butyrate-MEC: pre-acclimated to butyrate; CSFE-MEC: switchless-control (The applied voltages of each cycle were 0.8 V except for the first three cycles. Cycle 1: 0.5 V; Cycle 2: 0.6 V; Cycle 3: 0.7 V.). (Adapted from Li et al, 2017).

Acetate, when used as a pre-acclimation substrate for bio-anodes, also decreased the MECs performances in two other studies [138, 142]. In the first study, in addition to the acetate-condition, a multi-carbon mixture was used including glucose, ribose, glycine, cysteine and potassium hydrogen phthalate, in order to improve the treatment capacity and current output of electrochemically active bacteria during real wastewater treatment in fed-batch MFC [138]. Such multi-carbon mixture was used to promote the establishment of a consortium with a high metabolic versatility by favoring two main microbial communities: the fermentative &

electroactive bacteria. Concerning performances after cross-feeding with wastewaters, the multi-carbon mixture was the best strategy in terms of maximum current density produced compared to the acetate only acclimation and for four initial concentrations (52.8, 66.3, 80.8 and 105.8 mg COD.L⁻¹) (Fig. 1-20). Interestingly, it was observed that performance improvements were constant over a period of more than one month. In this study, it would have been interesting to analyze the microbial communities by NGS to observe the different fermentative bacteria that were favored by the multi-carbon mixture with regard to acetate, to know whether the latter could explain the improvement in current density.

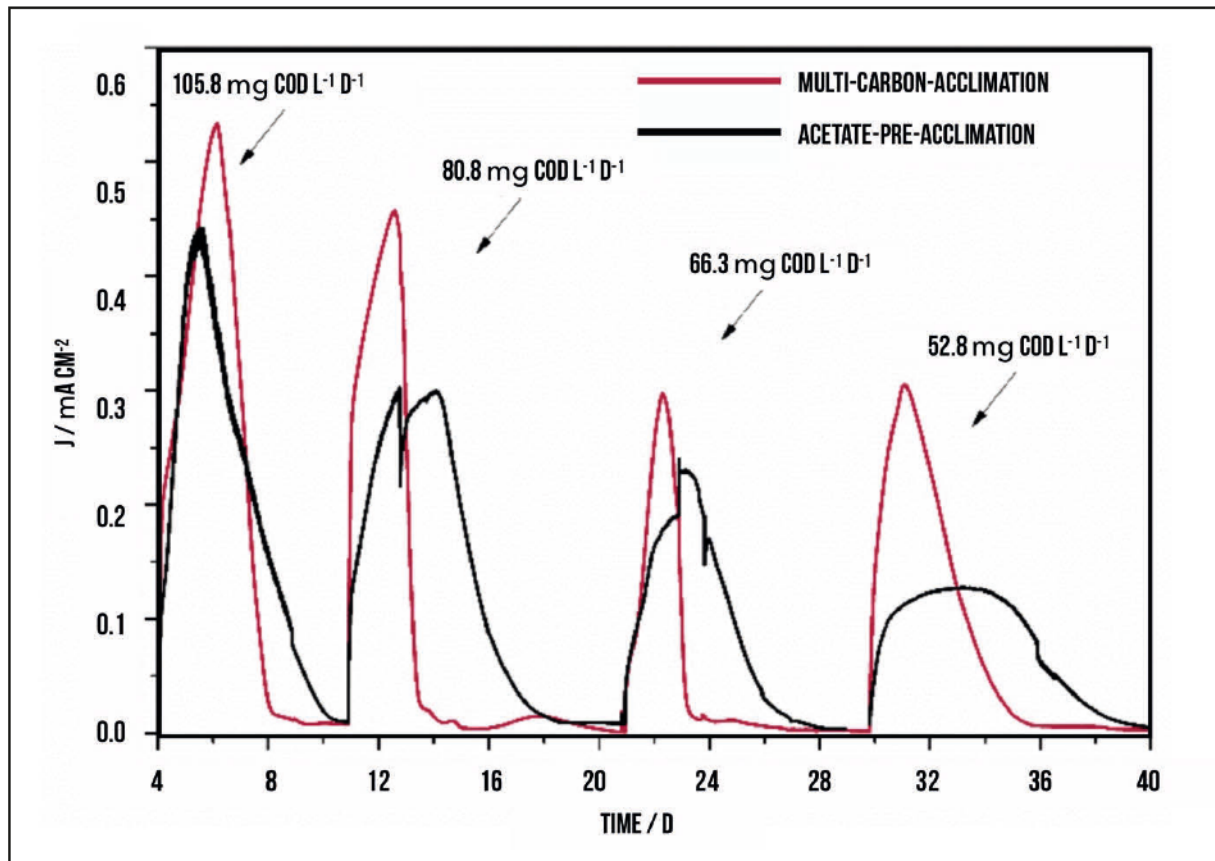


Fig. 1-20 Chronoamperograms (current density, j) from batch experiments of each preselected bio-anode degrading aliquots of the same real wastewater at decreasing loading rates. The loading rate of each cycle is given in chronological order (Adapter from Riedel et al, 2017).

In the second study, Park et al., (2017) use three substrate pre-acclimation strategies: (1) two step pre-acclimation with acetate and then glucose (acetate/glucose) before supplying domestic wastewater; (2) one step pre-acclimation with acetate before supplying domestic wastewater; and (3) direct supply of domestic wastewater without any pre-acclimation (switchless-control) in fed-batch mode MFC. After cross-feeding with domestic wastewaters, the acetate/glucose condition showed the best performances when compared to the two others [142]. In fact, power generation was improved by 50% and the CE by 24.1% with regard to the

switchless-control. The acetate condition showed the lowest performances with only 0.7 mA, against 0.9 and 1.4 mA in the switchless-control and acetate/glucose condition respectively (Fig. 1-21). According to a Fast-Unifrac ordination of the data, it was shown that consortia were significantly impacted, consistently with the pre-acclimation procedure. No convergence was observed, suggesting a long-term influence of the pre-acclimation techniques on the electroactive consortia. (Fig. 1-22).

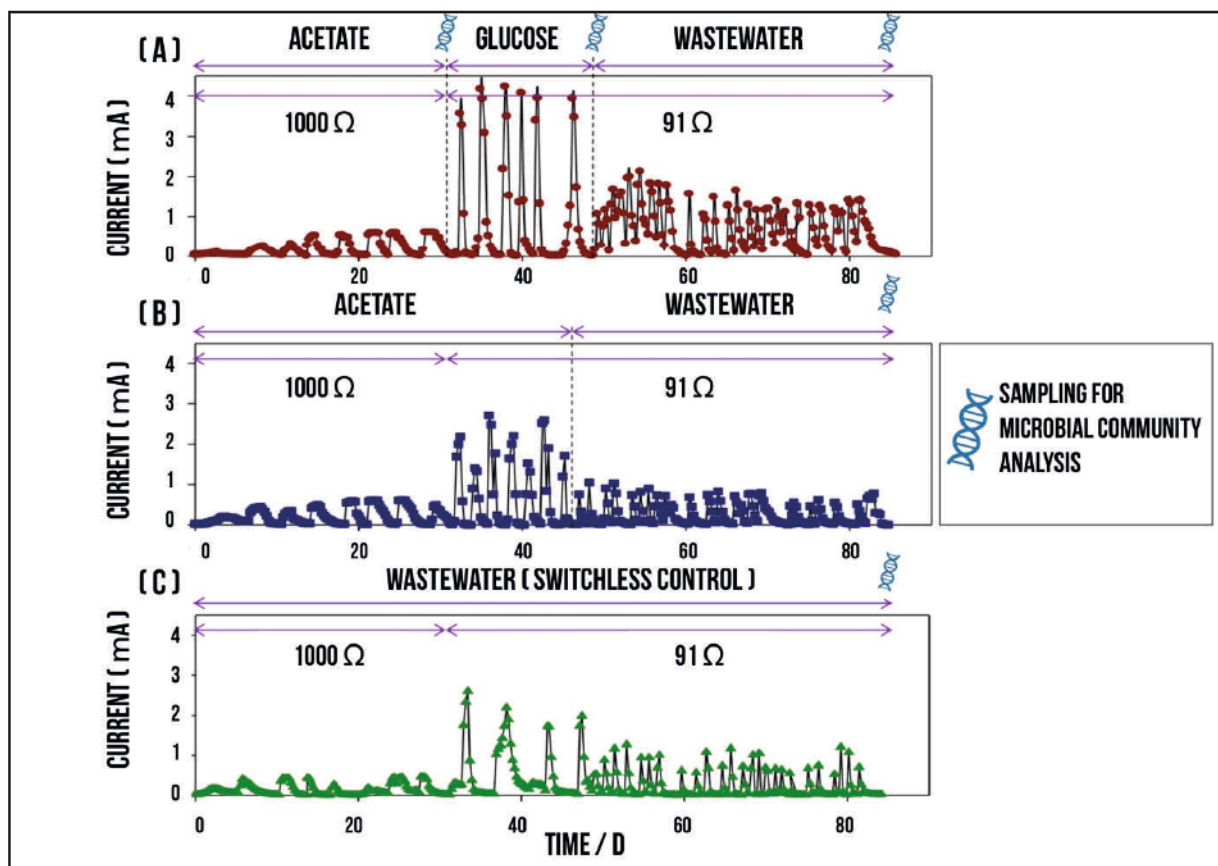


Fig. 1-21 Current generation indicated every five hours in air-cathode MFCs applied with (A) Acetate/Glucose ;(B) Acetate and (C) Switchless-control. (Adapted from Park et al. 2017).

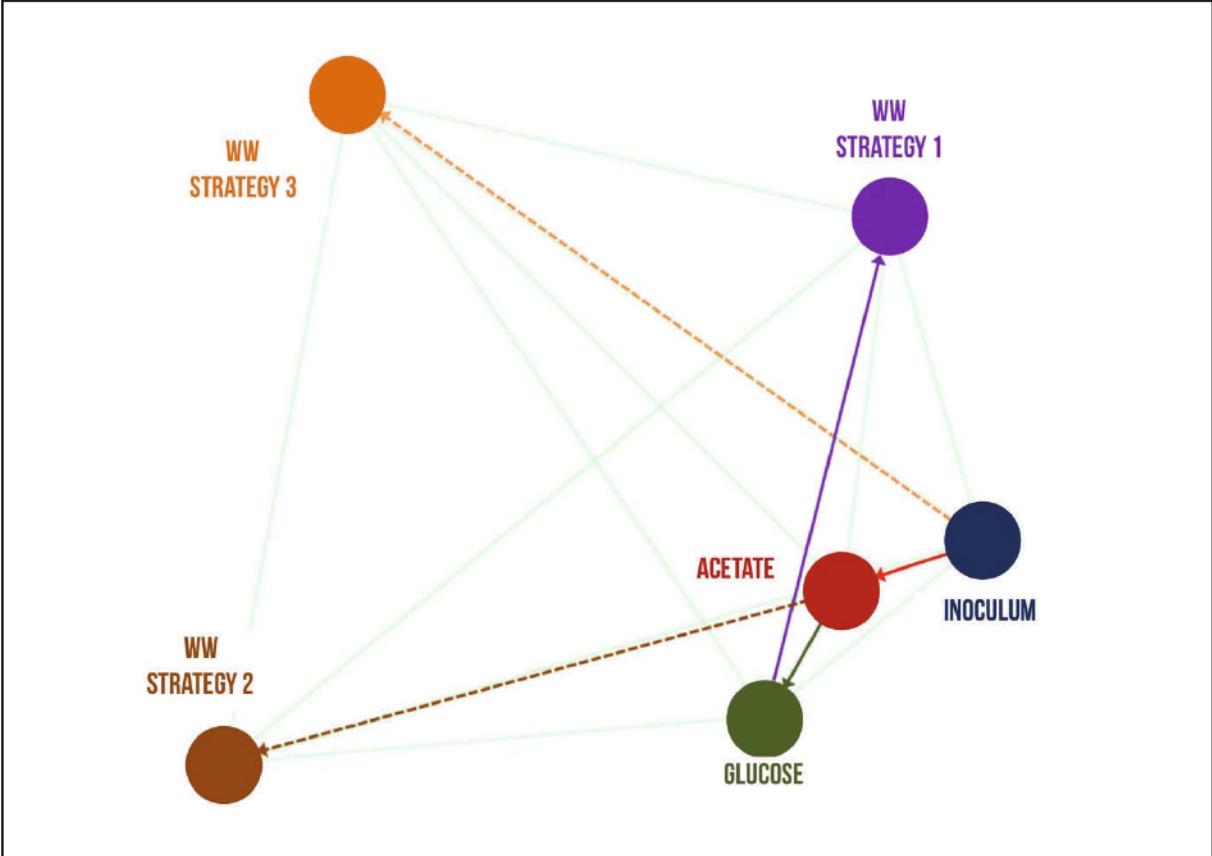


Fig. 1-22 Visualization of beta diversity using a principal coordinate based on a Fast Unifrac analysis. Samples mean inoculum and anodic biofilms at the end of substrate conditions; acetate, glucose, and domestic wastewater (WW). Arrows indicate change of microbial communities of each strategy: 1 (solid arrows), 2 (dotted dark brown arrow), and 3 (dotted light brown arrow). (Adapted from Park et al. 2017).

In summary, an analysis of these different studies shows that substrate-based pre-acclimation techniques can significantly improve the main performance indices in MXCs (Table 1-4). A variation in effectiveness of the substrate used for the acclimation as a function of the wastewater type could be observed, suggesting a need to adapt these techniques to each effluent. The observed trend was an improvement in the presence of fermentative substrates, such as glucose, butyrate and multi-carbon mixture, when compared to only acetate. Many

studies used acetate as starting substrate because it is rapidly converted to current, but it does not appear to be an efficient strategy for application on real wastewater. This indicates that, from an ecological point of view, it seems necessary to establish a syntrophy-based ecosystem: fermentative & electroactive consortia. The differences between pre-acclimated consortia structures, due to diverse fermentative bacteria, generate varied metabolic pathways, probably giving various performances. It is also essential to understand whether the microbial communities converge after cross-feeding in order to determine how the improvement will be long-term or short-term range and to know if it would be essential to pre-acclimate at specific frequencies to achieve long-term improvements.

1.4.2.2 Mix strategy

Another strategy called mix strategy was previously defined as when a simple substrate (or multi-substrates) or wastewater is added in mixture with either another wastewater or a simple substrate (or multi-substrates) (Fig. 1-9. B). The advantage of this method is the simplicity of implementation from an application point of view. It is therefore interesting to know whether substrate addition can influence communities and their related performance.

Liu et al., (2011a) studied the effect of three different simple substrates in fed-batch MFCs: acetate (440 mg.l⁻¹), fumarate (600 mg.l⁻¹), glucose (350 mg.l⁻¹) in mixture with urban wastewaters [156]. An unmixed-control was also set up (Fig. 1-9. C). A first step, known as pre-acclimation, consisted in mixing a wastewater and one simple substrate for ~525 hours in batch and then, the cross-feeding step, which consisted in transferring the pre-acclimated anode

to only wastewater for ~675 hours. The lag-phase was increased by up to +34% for the glucose condition compared to the unmixed-control. In terms of power production, the fumarate pre-acclimation was not effective with a decrease down to -45% (Fig. 1-23). These performances shown that the addition of these substrates to wastewater was inefficient considering these two important indices.

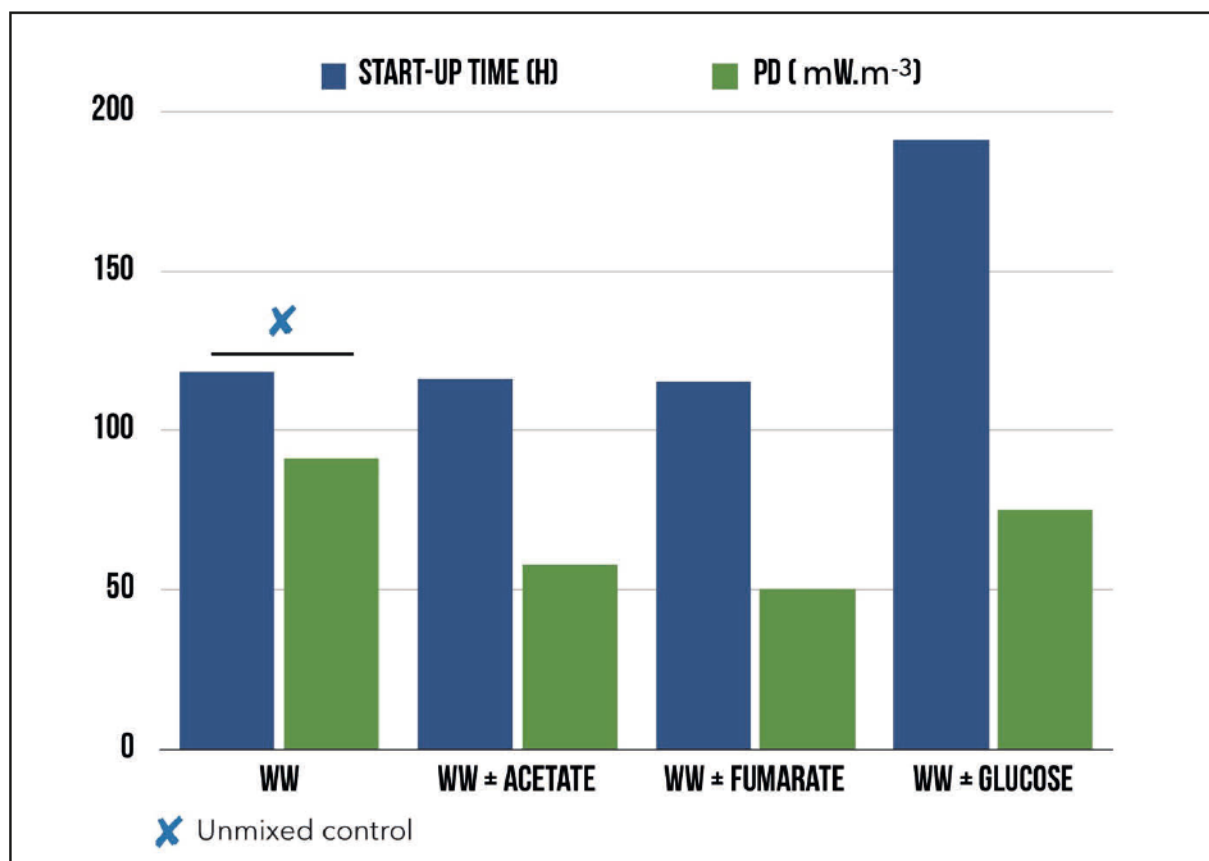


Fig. 1-23. Start-up time (hours) and power generation (mW.m⁻³) of acclimated MFCs when feed with wastewater. WW = Wastewater. (Data from Liu et al. 2011).

Concerning the community analysis, two main clusters were distinguished: pre-acclimated consortia and unmixed-control (Fig. 1-24). Therefore, the simple substrate addition does not significantly impact the community structure. The structural divergence is similar to

unmixed-control, suggesting that the experiment duration was the main selection factor. To verify this point, it would have been interesting to analyze the microbial diversity over time to observe the dynamics of community structure. In addition, it would be interesting to test this strategy with other wastewaters as it is easy to implement in the case of a larger scale application. Besides, it could be interesting to analyze again the community at long term to see if it became stable or it continuously varying over time.

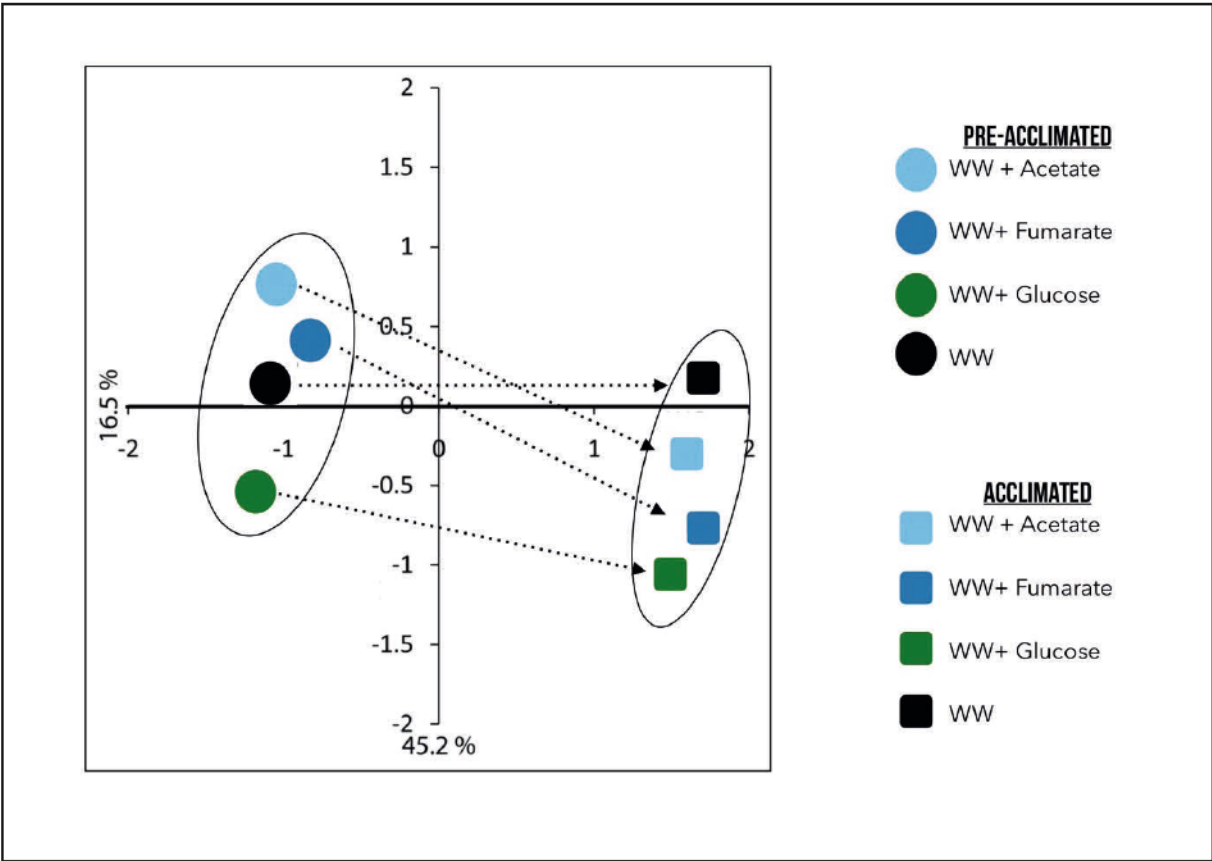


Fig. 1-24 PCA analysis showing the community shift between pre-acclimated and acclimated MFCs. WW = Wastewater. (Adapted from Liu et al. 2011).

Two different mixed pre-acclimation strategies were tested with the objective of treating industrial wastewater (IW) in batch-mode MECs [3]. Acclimation with domestic wastewater (DW) and acclimation with a 1:1 mixture of industrial and domestic wastewater (IW + DW) were compared to direct use of IW (no-acclimated control). Then, a cross-feeding was carried out and only industrial wastewater was used as substrate. DW/CF corresponds to the anode fed with domestic wastewater after cross feeding and IW+DW/CF corresponds to the anode fed with a 1:1 mixture after cross feeding. A 70.5% reduction of time per batch compared to the non-acclimated control (IW) was observed for the IW+DW/C condition which was the best strategy (Fig. 1-25). In this case, it would have been interesting to analyze the associated communities by NGS to identify the community variances between the conditions and understand the reasons for a decrease in time per batch (e.g. different fermentative bacteria).

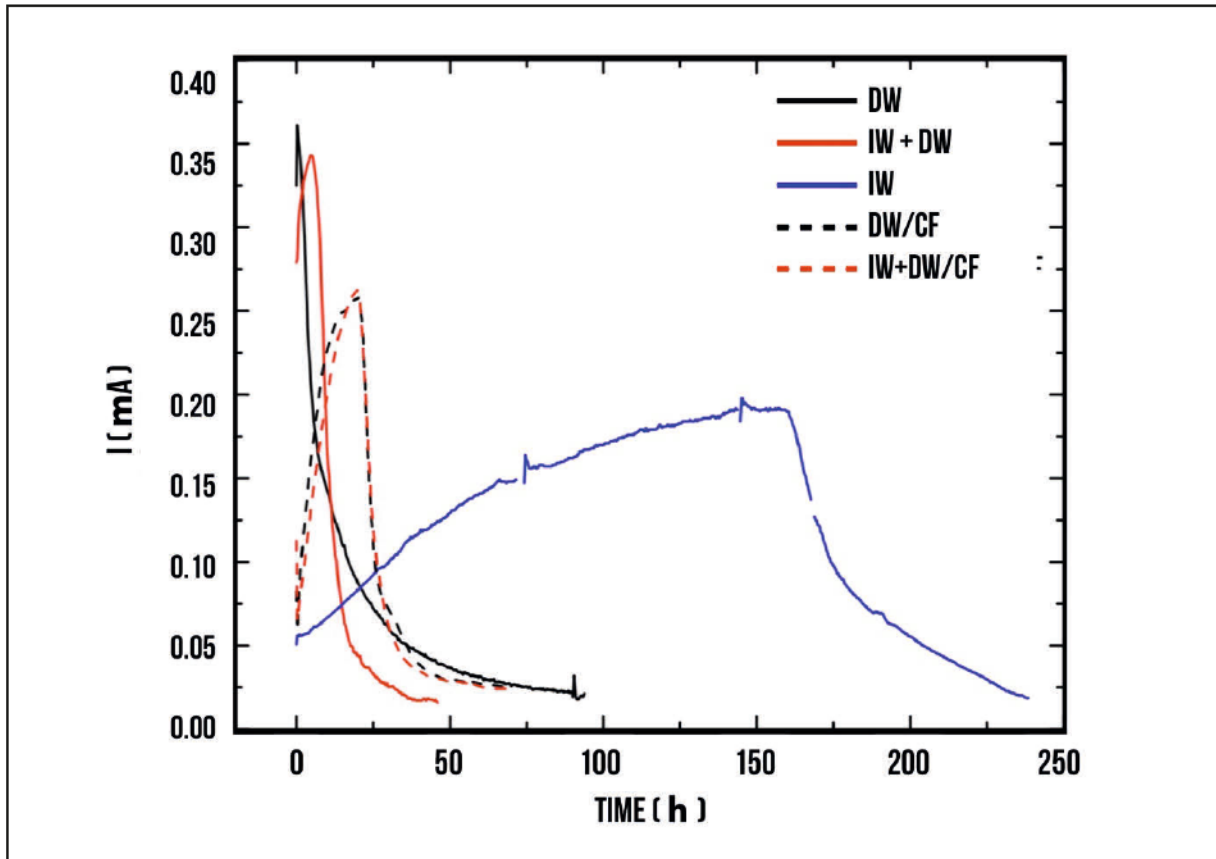


Fig. 1-25 Current profiles during a representative batch cycle in MECs operated with different wastewater samples. CF (Cross Feeding) = after transfer with industrial wastewater in substrate. (From Ivanov et al. 2013).

1.4.2.3 Disconnected pre-acclimation example of cheese whey

Finally, an original strategy was tested specifically to treat cheese whey. The pre-acclimation strategy consisted of first submerging an anode into cheese whey without connecting it (open circuit) and leaving it into the reactor without refreshing the medium. After a defined period (30 and 90 days), the reactor was switched to fed-batch MFC [157]. The aim was to promote the emergence of bacteria in contact with the anode, a conductive material, and

to determine whether the maturity of the biofilm (i.e. the incubation time) could be related to the power produced. A non-acclimated control was also set-up, in which the anode was directly immersed into the cheese whey effluent. After cross-feeding, the power output of the 30-day acclimation was not significantly different from the non-acclimated control being both around 30 W.m^{-2} , whereas 90-day acclimation lead to a power of 1800 W.m^{-2} (Fig. 1-26A). In addition, the 90-day acclimation current output was more stable over time (11 stable days) compared to the two other conditions (~4 stable days) (Fig. 1-26.B).

To better understand the probable reason for this performance difference from the same wastewater, scanning electron microscopy was carried out (Fig. 1-27). From the photo, clumps can be observed (cellular aggregates), but they were immature (irregular ovoid) on the 30 days-anode whereas they were more developed on the 90 days-anode (regular ovoid) and highly connected with many filaments. The formation of a more effective biofilm able to oxidize cheese whey substrates at the anode was favored by an increase of the acclimation time.

This example shows that even without being connected, an anode can promote the emergence of electroactive cells with filaments over time, but it seems necessary to wait for a long period (3 months) for the growth of the biofilm to achieve high performances. This study shows that the biofilm maturity is a very important indicator of system performances. Furthermore, it was demonstrated by Viridis et al (2014) that this factor was related to the biofilm thickness. Thus, biofilm thickness is an indicator of low performance, because if a biofilm is thicker than $100 \mu\text{m}$, it accumulates electrons (especially at the iron atom of cytochromes) forming a redox gradient with subsequent decrease of the electron flow [158]. Therefore, the development of highly connected cells within a thin biofilm would be required.

In addition, it would be interesting to know whether microbial species in the biofilm change over time or only the cells connections (e.g. e-pili) increased with the acclimation time leading to better performances. Taken together, these results pave the way for the strategy development of electroactive communities, taking into account not only the species present but also their interconnection level and the biofilm thickness using concomitantly NGS and microscopy analysis.

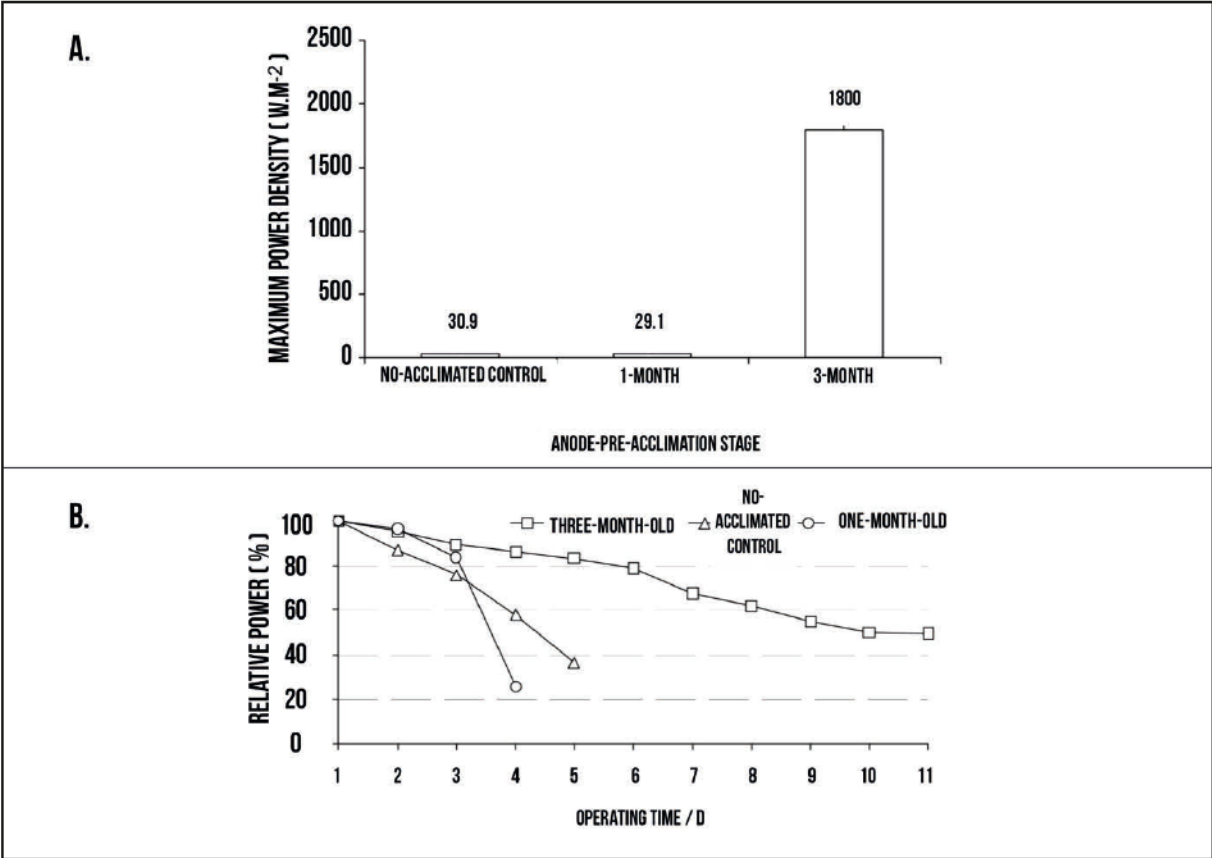


Fig. 1-26 A. Maximum power densities (W/m²) from reactors with the anodes subjected to different periods of biofilm build-up. In the no-acclimation control, the anode was directly used

without prior immersion in the whey. B. Relative power (%) in MFCs with different anode pre-acclimation procedures over 11-days period. (Adapted from Kassongo. 2011).

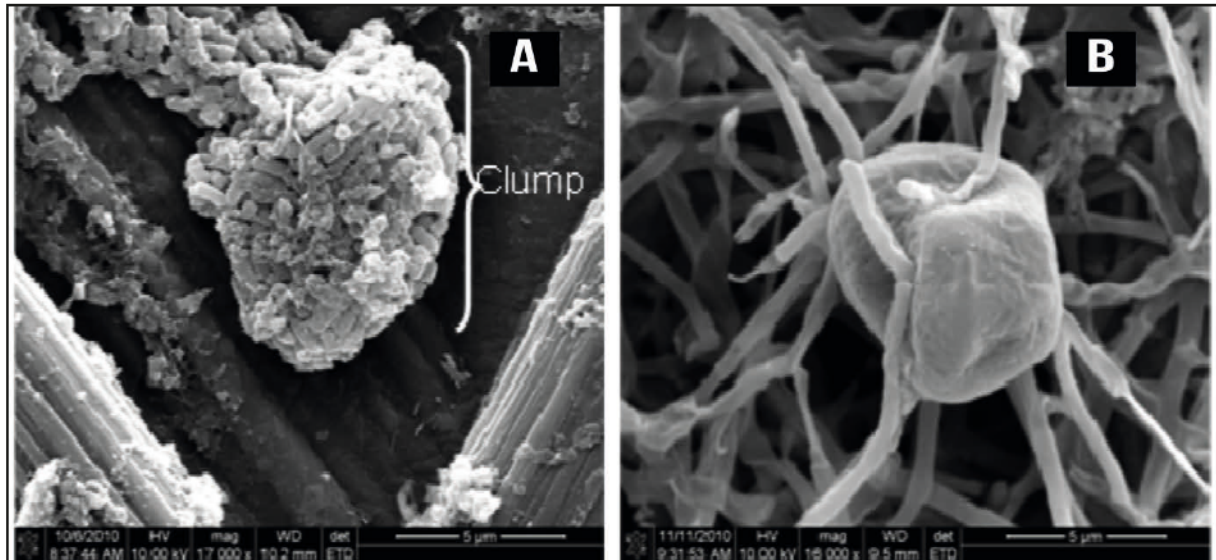


Fig. 1-27. Scanning electron micrographs of anode surfaces illustrated the morphologies of microorganisms on one- (A) and three-month old (B) anodes subsequent to incubations in open-circuit MFCs. (From Kassongo. 2011).

Table 1-4. Summary of the different wastewater substrate based pre-acclimation studies in MXCs. IW (Industrial Wastewater), DW (Domestic Wastewater), RW (Refinery Wastewater), Multi-carbon (glucose, (D)-ribose, glycine, cysteine, potassium hydrogen phthalate), Mix (Glucose, Butyrate, Propionate, Acetate), CSFE (Corn Stalk Fermentation Effluent). Controls: switchless-, unmixed- or no-acclimation-controls

MXCs	First substrates	Wastewater (switch mode)	Microbial analysis	Controls	Ref.
MFC	Glucose -> Lactose	Cheese whey	-	-	[152]
MEC	IW; DW; IW+DW	IW	-	No- acclimated	[3]

Chapter 1 Literature review

MEC	DW; DW+RW	RW	-	switchless	[153]
MEC	DW; Acetate	Cellulose fermentation	-	switchless	[4]
MFC	Glucose; Butyrate; Propionate; Acetate; Mix	Brewery	DGGE	switchless	[154]
MFC -> MEC	Acetate; Butyrate	CSFE	-	switchless	[155]
MFC	Multi-carbon; Acetate	UW	NGS	-	[138]
MFC	Acetate- >Glucose; Acetate	DW	NGS	switchless	[142]
MFC	Cheese Whey- 30d; Cheese Whey-90d WW+acetate:	Cheese whey	Scanning electron micrograph	No- acclimated	[157]
MFC	WW+fumarate; WW+glucose	WW	DGGE	unmixed	[156]

Conclusion

In this section, about fifteen studies on different substrate-based acclimation strategies have been reported. Among them, an overall trend was observed: acetate is the substrate which, in most cases, gives the lowest performances on more complex substrates when compared to other fermented substrates, such as glucose or sucrose. The growth of syntrophic fermentative bacteria enables the establishment of a syntrophy-based ecosystem (fermentation and electroactive) as preferred path. Syntrophic bacteria are capable of oxidizing many carboxylic acids (e.g. *Synergistaceae* & *Syntrophomonadaceae*) and consequently lead to a consortium with high metabolic versatility, very effective in substrate-switching condition. Nevertheless, there are still many elements to be characterized such as the microbial species involved, their type of interaction and the fundamental reasons for a variation in performance. From an application point of view, it seems necessary to adapt the type of pre-acclimation according to the type of wastewater/effluent to be treated. Then, it would be interesting to know the time during which pre-acclimation is stable by setting up a long-term experiment and periodically analyze the communities by NGS. That would help to determine the convergence time between the acclimation strategies. In the future, it is necessary to acquire a better understanding of bacterial consortia with on the one hand a high resolution (with NGS techniques) and a robust experimental design (e.g. quadruplicates) in order to characterize the bacteria involved in the oxidation of fermentation products found in wastewater. Indeed, in the literature, studies provide few replicates, and often a brief analysis of communities (most of them are based on molecular fingerprint techniques such as DGGE). After this first stage, it would be essential to propose microbial ecosystem management techniques in order to improve system performance with defined substrates to test the effectiveness of the eco-engineering technique with a NGS

analysis of microbial communities, what is currently missing in the literature, to understand the reasons for the effectiveness or not of the proposed technique. Finally, it would be interesting to test eco-engineering technique with real wastewater with a successful scale up perspective.

The first objective of this thesis was to characterize the bacterial consortia present in MECs as a function of the electron donor. Acetate, lactate, butyrate and propionate were selected because they are present in many organic wastewater as fermentation products. The results of this first experiment were analyzed using NGS focusing on the potential electroactive bacteria according to the substrates (Chapter 2). Secondly, a core-microbiome study was established to determine bacteria in common whatever the substrate and what functions are brought to the communities (Chapter 3). Propionate has been recalcitrant over time, especially when mixed with acetate following the fermentation of lactate. Therefore, the second objective of this thesis was to overcome this recalcitrance by using substrate-based pre-acclimation techniques through switch strategy in MECs, with acetate, lactate and propionate as pre-acclimation substrates (Chapter 4). Finally, a pre-acclimation technique with different mixtures of three inocula (sludge from an industrial wastewater treatment plant, aerobic sludge from the aeration tank of the Narbonne wastewater treatment plant and EAB-enriched culture in acetate-fed air-cathode MFC) and without mixing was investigated in MFCs for the treatment of industrial wastewater rich in lactic acid. The objective was to determine the best inoculation strategy with regard to performance indices (CD, CE, Time per Batch) (Chapter 5).

2 Chapter 2. Microbial anodic consortia fed with fermentable substrates in microbial electrolysis cells: significance of microbial structures

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Avant-propos

Pour améliorer les MECs dans le cadre du traitement des eaux usées, il convient dans un premier temps de comprendre l'écosystème bactérien impliqué dans la conversion de la matière organique en courant électrique à partir d'eaux usées. Les eaux usées urbaines étant principalement composées d'acides organiques issus des processus fermentaires, les principaux ont été sélectionnés : l'acétate, le lactate, le butyrate et le propionate. L'utilisation séparée de ces donneurs d'électrons permet de simplifier l'écosystème étudié et d'identifier les consortia bactériens associés à chaque acide organique. L'objectif de cette étude était d'identifier les consortia microbiens anodiques en fonction du donneur d'électrons.

La synthèse bibliographique a montré deux lacunes principales concernant le mode d'expérimentation : (1) le nombre insuffisant de répliques nécessaires pour évaluer la reproductibilité expérimentale et mener des analyses statistiques robustes ; (2) l'utilisation principalement des techniques d'écologie microbienne à empreintes moléculaires ayant une faible résolution phylogénétique.

L'expérience présentée dans ce chapitre a été conçue de façon à améliorer ces deux aspects, avec d'un côté la mise en place de quadruplicats par acide organique et de l'autre une analyse des communautés par séquençage haut-débit à haute résolution phylogénétique (possibilité d'associer les OTUs jusqu'à l'espèce).

ABSTRACT

Microbial community structure of anodic biofilms plays a key role in bioelectrochemical systems (BESs). When ecosystems are used as inocula, many bacterial species having interconnected ecological interactions are present. The aim of the present study was to identify these interactions for the conversion of single substrates into electrical current. Dual-chamber reactors were inoculated with activated sludge and fed in batch mode with acetate, lactate, butyrate and propionate at 80 mM^{e-} equivalents in quadruplicate. Analyses of biofilms and planktonic microbial communities showed that the anodic biofilms were mainly dominated by the *Geobacter* genus (62.4 % of the total sequences). At the species level, *Geobacter sulfurreducens* was dominant in presence of lactate and acetate, while *Geobacter toluenoxydans* and *Geobacter pelophilus* were dominant with butyrate and propionate as substrates. These results indicate for the first time a specificity within the *Geobacter* genus towards the electron donor, suggesting a competitive process for electrode colonization and the implementations of syntrophic interactions for complete oxidation of substrates such as propionate and butyrate. All together, these results provide a new insight into the ecological relationships within

electroactive biofilms and suggest eco-engineering perspectives to improve the performances of BESs.

Keywords: Anodic consortia – Microbial Electrolysis Cells – Fermentable substrates – Ecological relationships

2.1 Introduction

The massive use of fossil fuels has increased pollution with major climatic disruptions which implies the absolute necessity of developing renewable energies. New solutions need now to be considered to produce clean energy, and sustainable hydrogen is a good alternative for future transportation. Among the technologies able to generate H₂, microbial electrolysis cells (MECs) constitute a very promising solution. In MEC, the organic matter contained in wastewaters is oxidized at the anode in CO₂, electrons and protons by specific bacteria named electroactive bacteria (EABs). Electrons cross then the electrical circuit up to the cathode where they combine with protons to form hydrogen. This biological-assisted reaction requires a lower voltage (0.2-0.8 V) than water electrolysis (1.23-1.8 V) [159, 160]. Significant advances have been recently made to improve MECs performances through the increase of the current density (CD) and coulombic efficiency (CE), two essential parameters for future large-scale implementation [161]. While many parameters (e.g., architecture, materials) are known to affect MECs performances, anodic biofilm, as catalyser, is the fundamental parameter to be optimized for converting the electrons' flow to the electrode [162]. These biofilms are mostly composed of EABs able to use an anode as final electron acceptor [129]. Such electronic

transfer can result from either a direct contact with the anode through redox active proteins (short range), or e-pili (long range), or an indirect transfer through soluble electronic shuttles [30]. During the oxidation of organic compounds (e.g., volatile fatty acids), it is necessary to maintain a low partial pressure of hydrogen or a low concentration of formate to make the chemical reactions thermodynamically favourable [163]. This implies a critical interdependence between a producer and a consumer, so, called syntrophy [164]. Thus, for a complete conversion of fermentable substrates (e.g., glucose, propionate, ethanol) to electrons, fermenters produce intermediate compounds such as hydrogen, formate or acetate which are then used by EABs to generate electrical current [95]. Some EABs such as *Geobacter metallireducens*, can even convert directly fermentable substrates into electrons without syntrophic partners [63]. The numerous combinations of these interactions make the electroactive ecosystems still poorly understood.

One way to identify efficient anodic bacteria is to characterize the bacterial community composition of the anodic biofilms in relation to the MEC performances (CD & CE) [7]. Many substrate-specific EABs could potentially improve MECs performances. These EABs are efficient because they significantly contribute to the conversion of specific substrates into current. A great diversity is commonly observed together with the predominance of EABs or others metal-reducing bacteria [16, 92]. Among the already well known EABs, *Geobacter sulfurreducens* is often found dominant in ecosystems fed with either acetate or lactate as sole electron donor [11, 12]. Concerning propionate, the presence of *Geovibrio ferrireducens* was revealed by DGGE in microbial fuel cell (MFC) [13]. In the same study, *Pelomonas*

saccharophila was found as major DGGE-band when butyrate was the sole electron donor.

However, little information exists in the literature on the microbial structure of bioanodes.

The objective of this study was to determine the selection of substrate-specific microbial communities in MECs and bacteria directly related to electron fluxes. For that, four different substrates, acetate, lactate, propionate and butyrate were separately used as sole electron donor in quadruplicate. Indeed they are the main breakdown products produced by fermentative bacteria in wastewater treatment [133].

2.2 Materials & Methods

2.2.1 Inoculum

The microbial inoculum used in this work was sampled from the aeration tank of the Narbonne wastewater treatment plant (11100, France). The latter was freshly used without storage at 10% v/v.

2.2.2 Operating of the MECs

All chemicals were of analytical or biochemical grade and were purchased from Sigma-Aldrich. All potentials provided in this manuscript refer to the SCE reference electrode (KCL 3.0 M, +240 mV vs. SHE, Materials Mates, La Guilletière 38900 Sarcenas, France). All media prepared were adjusted to pH=7, flushed with high-purity N₂ gas (purity ≥ 99.995 %, Linde, France) for at least 30 min using air injection cannula. Bioelectrochemical experiments were conducted under potentiostatic control (BioLogic Science Instruments, France) with EC-Laboratory v.10.1 software and strictly anaerobic. All incubations were placed in a water bath at 37°C. A magnetic stirrer rotating a 350 rpm to homogenize the mixture. MEC tests were performed in quadruplicate with anodic potential fixed at +210 mV vs SCE.

2.2.3 Microbial electrolysis cell set up

The electrochemical system used for this experiment corresponded to a two-chambers cylindrical microbial electrolysis cell to avoid the diffusion of hydrogen from the cathode to the anodic compartment. Each chamber had a working volume of 900 mL. The anode was composed of a 2.5 cm x 2.5 cm x 0.12 cm carbon plate (Mersen S.A, France), screwed onto a 2-mm diameter titanium rod (T1007910/13, Goodfellow SARL, France). The cathode was made of a plate of 16 cm² of 90% Platinum and 10% Iridium mesh (Heraeus PSP., France). The MECs were hermetically sealed with silicone and stainless steel ring at each chamber. Both chambers were separated with an anion exchange membrane (AEM, Fumasep FAA, FuMA-Tech BWT GmbH, Germany). Batch was the operational mode for each experiment. When the current density (A. m⁻²) is close to half the maximum current density, the MEC has been stopped and the electroactive biofilm collected. This value was chosen to sample a still active biofilm.

2.2.4 MEC Medium

The medium in the anodic chamber (per litre of water) was as follows: 0.5 g K₂HPO₄, 2.0 g NH₄Cl, 7.6 g MES buffer, 0.2 g yeast extract, 12.5 mL trace metal element solution 141 (DSMZ), 2.11 g Sodium 2-bromoethanesulfonate (2-BES) to inhibit methanogens. The cathodic medium (per liter of water) contained 0.5 g K₂HPO₄, 7.6 g MES buffer and 12.5 mL trace metal element solution 141 (DSMZ). Acetate, lactate, propionate and butyrate were separately used in anodic compartment as unique electron donor at a concentration of ~80 m e⁻ eq.

2.2.5 Analytical Methods

Concentrations of acetate, propionate, butyrate and lactate were measured by HPLC with a refractive index detector (Waters R410). First, samples were centrifuged at 13,500g for 15 min and then supernatants were filtered with 0.2 µm syringe filter. HPLC analysis was performed at a flow rate of 0.4 mL/min on an Aminex HPX-87, 300 x 7.8 mm (Bio-Rad) column at 35°C with H₂SO₄ (4 mM) as mobile phase. For each batch, the planktonic part was sampled after inoculation as the starting point, constituting the inoculum samples. At the end of each batch, the planktonic part was collected and constituted the bulk samples and the anodic biofilm was harvested with a blade. These three types of samples (Inocula, bulks and biofilms) were centrifuged at 13,500g for 15 min and the pellet was stored at -20°C prior to microbial community analyses.

2.2.6 Microbial Community Analysis

DNA extraction was carried out with QIAamp fast DNA stool mini kit in accordance with the manufacturer's instruction (Qiagen, Hilden, Germany). DNA extraction was confirmed using Infinited 200 PRO Nanoquant (Tecan Group Ltd., Männedorf, Switzerland). Amplicons from the V3 to V4 regions of 16S rRNA genes were amplified with bacterial forward 343F 5'-TACGGRAGGCAGCAG-3'; (Liu et al., 2007) and reverse 784R 5'-TACCAGGGTATCTAATCC-3'; (Anderson et al., 2008) primers. Adapters were added for multiplexing samples during the second amplification step of the sequencing. The PCR mixtures (50 µl) contained 0.5 U of Pfu Turbo DNA polymerase (Stratagene) with its corresponding buffer, 0.5 mM of each primer, 200 mM of each dNTP and 10 ng of genomic

DNA. Reactions were carried out in a Mastercycler thermal cycler (Eppendorf) as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min. The size and amount of PCR products were measured using a Bioanalyser 2100 (Agilent). The community composition was evaluated using the MiSeq v3 chemistry (Illumina) with 2 x 300 bp paired-end reads at the Genotoul platform (www.genotoul.fr). Sequences were retrieved after demultiplexing, cleaning, and affiliating using Mothur [165]. All sequences were submitted to Genbank under accession numbers MG238597 - MG241108.

2.2.7 Quantitative PCR (qPCR)

PCRs were prepared using 96-well real time PCR plates (Eppendorf, Hamburg, Germany) and Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). After, 6.5 µl of Express qPCR supermix with premixed ROX (Invitrogen, France), 2 µl of DNA extract with three appropriate dilutions, 100 nM forward primer F338-354 (5'-ACTCCTACGG GAGGC AG-3'), 250 nM reverse primers R805-785 (5'-GACTA CCAGG GTATC TAATC C-3'), 50 nM TaqMan probe and water were added to obtain a final volume of 12.5 µl for all analyses. A first incubation of 2 min at 95°C followed by 40 cycles of denaturation (95°C, 7 s; 60°C, 25 s) was performed. From each assay, one standard curve was generated by using tenfold dilution in sterilized water (Aguettant Laboratory, Lyon, France) of a target plasmid (Eurofins Genomics, Germany). The initial DNA concentrations were quantified using the Infinite 200 PRO NanoQuant (Tecan, France).

2.2.8 Electron balances

To estimate the electronic balances in each experiment, the distribution of electron in milli e^- equivalent ($m e^- eq$) in the MECs was monitored by determining the electron donor conversion (acetate, propionate, lactate and butyrate) to various electron sinks (current, propionate, acetate, lactate, butyrate). Coulombic efficiency (CE) was estimated as the percentage of electrons that have passed through the circuit in a single batch test divided by the amount of electrons available after substrate oxidation [1].

2.2.9 Statistical Analysis

All statistical analyses were carried out in R version 3.2.3 (R core Team 2014). The differences between current densities and coulombic efficiencies between the conditions were tested with one-way ANOVA followed by Tuckey's HSD post-hoc test with a statistical significance (P -value) < 0.05). Weighted-UniFrac distance-based PCoA ordination was used to represent inter-samples distances with phyloseq package [166]. The Monte Carlo simulation showed significant difference between microbial composition according to the substrates and sample types (Inocula, Bulks and Biofilms) with P -value of 0.001.

2.3 Results & discussion

2.3.1 Influence of single substrates on coulombic efficiency and current density

First, MECs performances were substantially influenced by the type of substrate. Regarding the current density (average of the maximum current densities), acetate- and lactate-fed MECs were the most efficient systems with a current density of 2.28 ± 0.62 and 2.44 ± 0.71 $A.m^{-2}$ respectively, in contrast to butyrate and propionate fed-MECs (1.45 ± 0.32 and $1.11 \pm$

0.22 A.m⁻² respectively) (Fig. 2-1-a). From these results, it was concluded that acetate and lactate were more rapidly converted into current. Acetate is a substrate which commonly produces high current densities in mixed cultures [2]. Since acetate accumulated after lactate fermentation (concomitantly with propionate, Fig. 2-5), it was not surprising to find similar current densities between acetate- and lactate-fed MECs. As observed in other studies, propionate and butyrate-fed MECs had the lowest current densities [90, 92].

In terms of coulombic efficiencies (CE), (average of the 4 MECs per substrate), acetate-fed MECs showed the highest values ($84.7 \pm 1.43\%$) as widely observed in other studies (Fig. 2-1-b) [13, 18]. Lactate-fed MECs had an average CE of $77.5 \pm 4.55\%$, indicating efficient electron recovery, probably due to the production of acetate as main fermentative product. Propionate- and butyrate-fed MECs had no-significant difference with respect to the CE (74.6 ± 1.72 and 70.2 ± 1.49 respectively). Based on these results, it appears that acetate was effectively converted to current. However, the addition of 2-BES that inhibits electron leakage to methane improved the coulombic efficiency for all the substrates tested in this study.

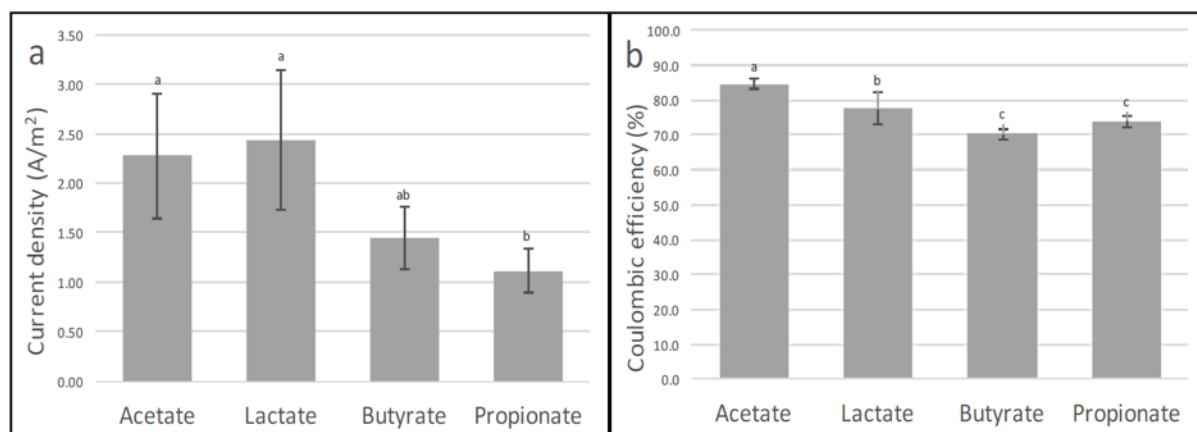


Fig. 2-1 Performance of MECs (average of the batch cycles of the duplicate (n=4) reactors in term of a current density of the maximum current density and b coulombic efficiency. Samples with the same letter (a, b or c) have no significant difference.

2.3.2 Electron distribution at the end of the batch experiments

Except for lactate-MECs, electron balance at the end of the batch tests revealed that current was the main electron sink (Table 2-1). Metabolite concentrations over time are shown in Fig. 2-5 & Fig.S 1-3. Nevertheless, most of the electrons remain present in the substrates (except for lactate), since the experiments were stopped when the current density was decreasing and close to half of the maximum peak. Concerning the lactate-fed MECs, the main end product was propionate 59.16 ± 9.34 %. This metabolite, together with acetate, is a co-product of the lactate fermentation. No significant difference was observed between all conditions probably due to a high intra-sample variability (standard deviation, Fig. 2-1-b) and the addition of methanogenesis inhibitor (2-BES) which prevents electron leakage to methane.

Table 2-1

Distribution of electrons at the end of the MEC batch experiments in quadruplicate. 100% = initial electron content in the substrates.

Electron sinks	Fraction of electrons at the end of MECs tests (%)			
	MECs Acetate	MECs Lactate	MECs Propionate	MECs Butyrate
Current	37.21 ± 14.34	31.88 ± 4.2	30.13 ± 6.02	34.36 ± 13.68
Acetate	56.24 ± 17.09	-	-	-
Propionate	-	59.17 ± 8.24	59.16 ± 9.34	-
Butyrate	-	-	-	51.20 ± 11.21
Unknown sinks	6.54 ± 2.34	8.94 ± 2.00	10.76 ± 3.33	14.43 ± 6.42

2.3.3 Analysis of the microbial communities

2.3.3.1 Microbial diversity analysis

Principal Coordinate Analysis (PCoA) based on weighted-UniFrac distance matrix was used to represent the differences of microbial communities between the inoculum (corresponding to the ‘bulks’ at the beginning of each batch test) and the anode and bulk samples. Three clear and significantly distinct clusters (P -value < 0.05) regardless to the electron donor are observed in the PCoA plot (Fig. 2-2-a). Axis 1 represents 62.3% of the variance and allows to distinguish inocula, biofilms and bulks while axis 2 represents 18.9% of the total variance. To determine the differences in diversity between these three clusters and the electron donors, a Shannon index was calculated (Fig. 2-2-b). The Shannon index gives access to the specific diversity of each samples according to the number of species (species richness) and their distribution (specific equitability). Among the three clusters (Fig. 2-2-a), a significant difference was found

between the Shannon indexes of the inocula, which had the highest diversity (5.57 ± 0.03), the bulks samples, having an average diversity (4.03 ± 0.22), and the anodic biofilms which had the lowest diversity (1.96 ± 0.16) whatever the substrate. The Butyrate-4 bulk sample had the lowest bulks' diversity (Fig. 2-2-a) which likely explained its presence close to biofilm's cluster on PCoA plot (Fig. 2-2-b). Moreover, the amount of 16S rRNA copies number, that is related to the cell number in each sample, is presented in Fig. 2-3. Here, inocula samples contained the highest copy numbers with an average of $2.95 \cdot 10^{12} \pm 3.69 \cdot 10^{11}$ copies of the 16S rRNA gene. At the end of experiments in bulks a significant decrease of the 16S rRNA copies number ($1.83 \cdot 10^{11} \pm 1.28 \cdot 10^{11}$) was observed, which indicates a cell mortality, probably due to the lack of soluble electron acceptors. Under these conditions, only some taxa were able to survive, which could explain the decline of the microbial diversity over time (Fig. 2-2-b). Concerning the biofilm samples, a number of $3.78 \cdot 10^{10} \pm 1.08 \cdot 10^{10}$ 16S rRNA copies was observed at the end of the experiments. This clearly indicate a cellular growth on the anodic surface. The low diversity of the biofilms (1.96 ± 0.16) compared to the bulks (4.03 ± 0.22), suggests that the growth was very selective. Therefore, the anode appears to be a very selective ecological niche for bacterial communities, probably due to the specific ability to form a biofilm and extracellular electron transfer to grow, both of which being two important ecological factors that lead to a significant decrease in diversity.

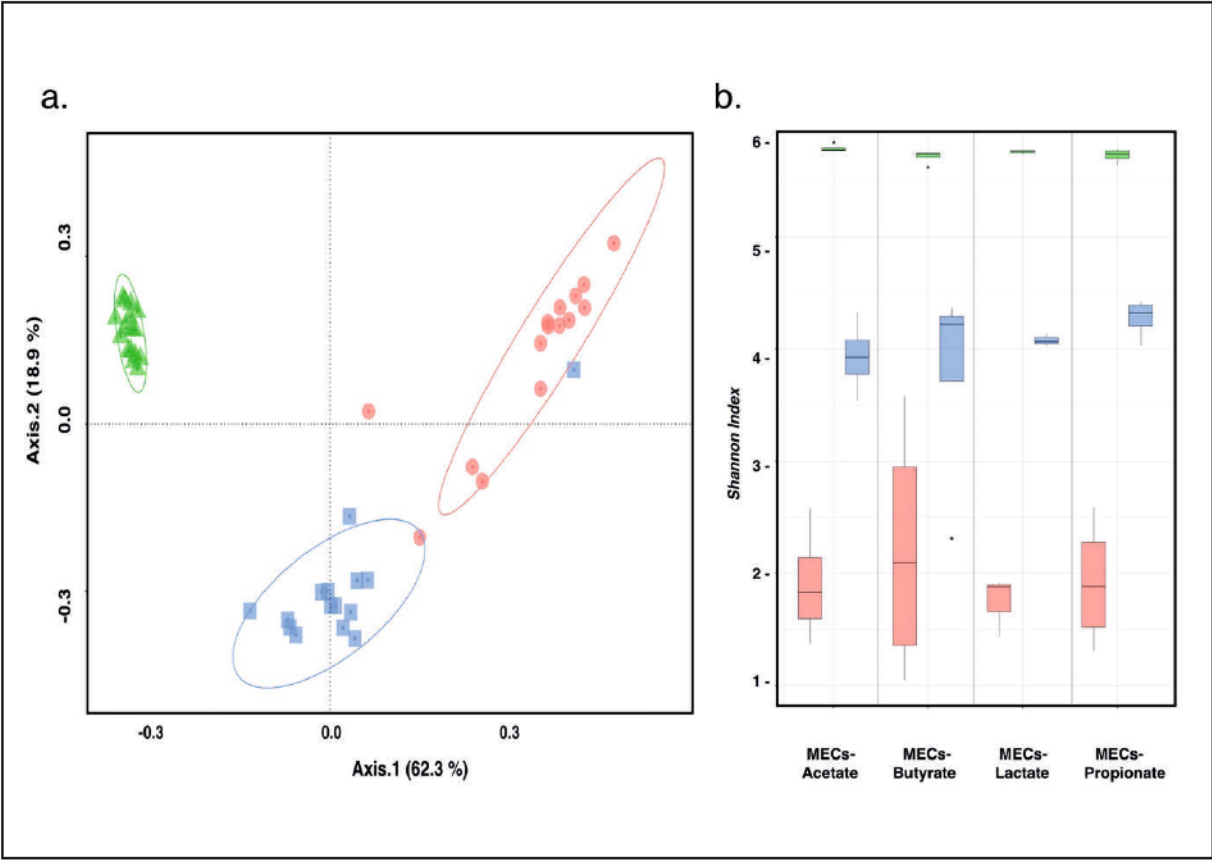


Fig. 2-2 a. Principal coordinate analysis (PCoA) based on weighted-UniFrac distance matrix showing the microbial distribution pattern for all substrates between inocula (\blacktriangle green triangles), bulks (\blacksquare blue squares) and biofilms (\bullet red points) samples. Clusters were defined by significance difference calculated by the permutation test ($n=9999$, $P.value=0.001$) b. Shannon index of microbial communities according to MECs-substrates and sample types (\blacksquare Inocula, \blacksquare Bulks and \blacksquare Biofilms).

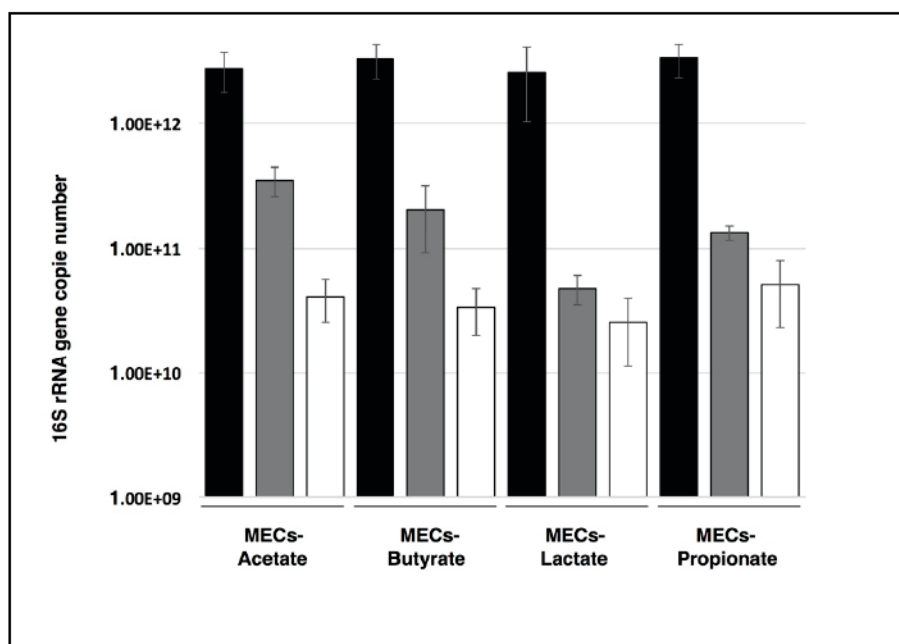


Fig. 2-3 Average of qPCR measurements per sample types (Inocula ■, Bulks ■ and Biofilms □) in the number of 16S rRNA gene copies.

2.3.3.2 Anodic microbial communities according to the substrates

High-throughput sequencing of 16S rRNA gene was used to characterize the bacterial communities of anodic biofilms and bulk samples from the sixteen MECs at the end of the experiments. 16S rRNA gene library had 1,957,962 high-quality reads (average length ~404 bp) after treatment (denoising, quality filtering and removal of chimeric sequences). Lactate-4 sample, composed of OTUs present at less than 3% was excluded from the analysis due to poor read sequencing quality. The sequences were assigned to OTUs with a $\geq 95\%$ sequence identity threshold. The classification with sequence identity of the bacterial communities is provided in Supplementary Information (Table S 1).

All biofilms whatever the substrate were dominated by members of the *Geobacteraceae* family, representing 62.41% of biofilms' sequences (Fig. 2-4). The *Rickenelaceae* family was also present in all biofilms samples at 10.89% and 12.61% in propionate-3 and acetate-3 MECs respectively. This family is represented by the *Blvii28* wastewater sludge group (OTU 9 & 54), known to be strict anaerobic fermenters [167]. The *Deferribacteraceae* family is mainly present in 2 samples, propionate-1 and butyrate-4 at 25.87% and 14.23% abundance respectively. This family is represented by *Selenovibrio woodruffi* (OTU 15). Interestingly, *S. woodruffi* can only use acetate as electron donor [168]. To explain its presence in biofilms, it could be involved in acetate oxidation generated by propionate or butyrate fermentation by syntrophic interactions. Selenate and arsenate, its known terminal electrons acceptors, are not available in the culture media. Therefore, it would be interesting to determine whether this bacterium has the ability to use the anode as final electron acceptor. The 'Others' category represents all OTUs with an abundance of less than 3% in all biofilms.

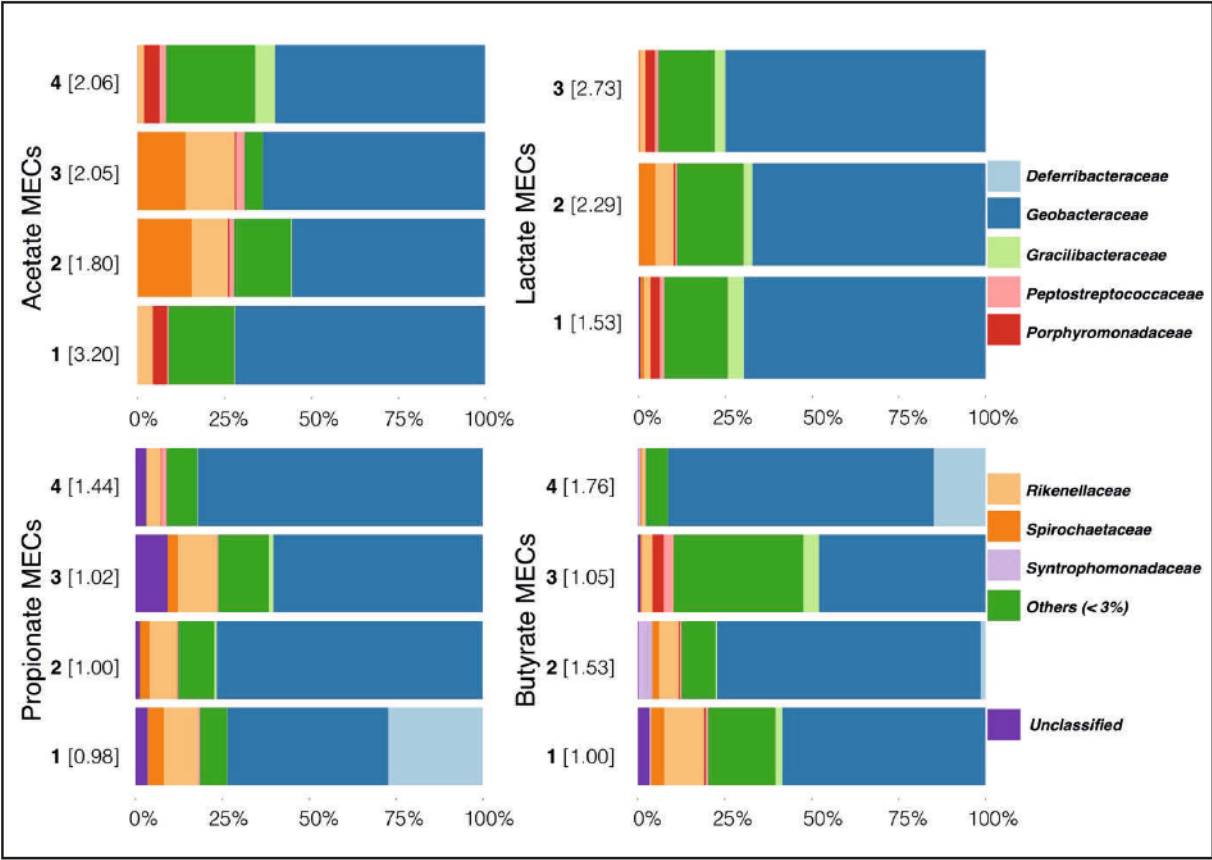


Fig. 2-4 Relative taxa abundances at Family level in anodic biofilms by MECs-type. Numbers (1-4) in bold are specific to the replicate (except Lactate-MEC 4). Numbers in square brackets correspond to the peak current density (A.m⁻²) of the sample.

2.3.3.3 Analysis of Geobacter species according to the substrates

As previously observed, the *Geobacteraceae* family was dominant in all biofilms. Species of this family have a well-known metabolism with high capability of exoelectrogenesis [169]. For better understanding the metabolic pathways and ecological interactions, the balance between *Geobacter* species was analysed. At this level, it showed a specific relative distribution according to the substrate within *Geobacter* species (Fig. 2-6).

Geobacter sulfurreducens (OTU 1) was the main *Geobacter species* in acetate, lactate and butyrate-fed MECs (except Butyrate-4) with a balance of $96.03 \pm 6.1\%$, $95.84 \pm 4.4\%$ and $64.55 \pm 30\%$ respectively. This species was also present at 39.80% in Propionate-3 MEC. *Geobacter toluenoxidans* (OTU 2) was dominant in propionate-fed MECs (except Propionate-3) at $78.51 \pm 18\%$ and replicate-4 of butyrate-fed MEC (97.78%). *Geobacter metallireducens* (OTU 5) was the second most abundant species in Propionate-1 and -3 MECs ($38.20 \pm 2\%$) and was also present at 9.33% and 21.24% in Acetate-4 and Butyrate-3 MEC respectively. *Geobacter pelophilus* (OTU 12) was dominant in Butyrate-1 sample (52.85%) and second most abundant in Butyrate-3 sample (23.53%) and at 7.05% in Lactate-2 MEC.

These results suggest that when acetate was in solution as main substrate or co-product i.e. Acetate- and Lactate-fed MECs (Fig. 2-5 & Fig. S-1), *G. sulfurreducens* predominated within the *Geobacter* genus. Indeed, this EAB is a well-known electroactive microorganism able to oxidize formate, H₂, lactate and acetate with the anode as sole terminal electron acceptor [170]. It could therefore be directly involved in acetate oxidation at the anode. *G. pelophilus* and *G. metallireducens*, which are also able to convert acetate into current were found in a minority in Acetate- and Lactate-fed MECs [171, 172]. Consequently, with regard to acetate as electron donor, competitive relations likely took place for anode colonization within the *Geobacter* genus. The competitive property of *G. sulfurreducens* leading to its predominance in the ecosystem has also been observed in a synthetic consortium [173]. In lactate-fed MECs, acetate was quickly oxidized (~4-6 days) while propionate was not degraded (Fig. 2-5). As observed in Fig. 2-4, these biofilms were dominated by *Geobacteraceae* family where *G. sulfurreducens*

was predominant (Fig. 2-6). Since the latter is unable to oxidize propionate, its presence caused a 'barrier effect' preventing the presence of other EABs able to oxidize propionate. Indeed, *G. toluenoxydans* was predominant with propionate as electron donor (and in Butyrate-4 MEC). It is able to oxidize many substrates such as acetate, propionate and butyrate by reducing ferrihydrite or ferric citrate [174]. The present OTU appears to be involved in propionate and butyrate conversion into current. Interestingly, the predominance of *G. sulfurreducens* in three butyrate-fed MECs (1-, 2- and 3-replicates) and Propionate-3 MEC whereas it is unable to use these two substrates as electron donors suggests that, within these biofilms, syntrophic interactions occurred. Similarly, *G. pelophilus* was dominant and second most abundant in *Geobacter* genus in presence of butyrate (Butyrate-1 and 3 MECs). This species is able to use acetate, pyruvate, ethanol and formate as electron donors but not butyrate, which suggests a syntrophic relationship with butyrate-oxidizing bacteria such as *G. metallireducens* [172]. Moreover, the latter is also known to establish syntrophic relationships with other *Geobacter* species [175]. Concerning propionate-fed MECs, *G. metallireducens* was systematically found beside *G. toluenoxydans*. *G. metallireducens* seems to have reached a specialized ecological niche in the use of propionate in a multi-species exoelectrogenic biofilm community [173]. These two bacteria which use the same electron donor, propionate, could indicate a competitive relationship to the substrate with an unexplained predominance for *G. toluenoxydans*.

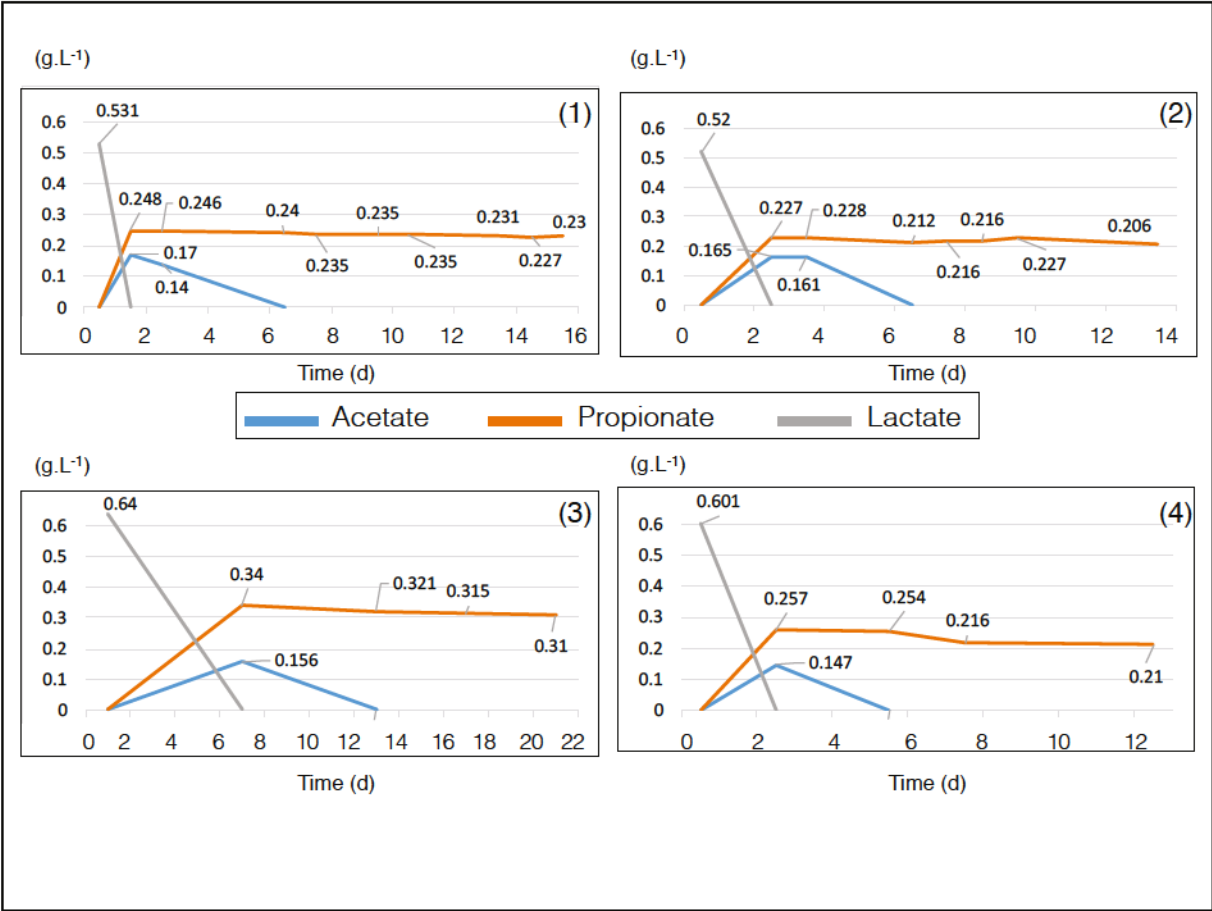


Fig. 2-5 Metabolite concentrations (g.L⁻¹) during lactate-fed MECs assays over time (d) according to the replicate numbers (1-4).

2.3.3.4 Analysis of bacterial communities according to MEC performance

Within the same substrate and similar physicochemical conditions, differences in peak current densities were observed (Fig. 2-4 & Fig. 2-6). It is therefore interesting to determine whether these differences can be explained by the composition of the bacterial communities. By this means, effective or ineffective species with respect to current densities can be determined. Firstly, for acetate- and lactate-MECs, there was a difference of 56.25 and 56.04 % between

the highest and lowest performance in CDs respectively (Fig. 2-4). With these substrates, *G. sulfurreducens* was dominant regardless of the CDs produced. Therefore, these differences were probably due to other bacterial families. In Acetate-MECs, the most efficient reactor (Acetate-1, 3.20 A.m⁻²) contains only 0.056% of *Spirochaetaceae* contrary to the least efficient (Acetate-2, 1.80 A.m⁻²) which contained 15.54%. This family is represented by *Treponema caldarium* (OTU 8) which is not able of using acetate directly as an electron donor but can be an hydrogen scavenger in ecosystems by oxidizing H₂ with CO₂ to produce acetate via the Wood-Ljungdahl (acetyl-CoA) pathway [176]. It would therefore be interesting to better understand its role in bio-anode to explain its ecological relationships and why this species seems to be ineffective regardless of the CDs produced. Concerning lactate-MECs, there is no difference in bacterial composition depending on performance (Fig. 2-4). In this case, minority bacteria, not well characterized in these systems, could play a role according to CDs. For propionate-MECs, a difference of 68.05 % in CDs is observed within the quadruplicate, with on one side Propionate-4 sample producing 1.44 A.m⁻² and on the other side Propionate-1 to 3 close to 1 A.m⁻². As with Acetate- and Lactate-MECs, these differences do not appear to be attributable to the *Geobacter* species distributions, as Propionate-4 and Propionate-2 contain a similar proportion of *G. toluenoxydans* (73 % and 67.45 % respectively) with a difference of 69.44% in CDs. Similarly, bacterial families have similar proportions regardless of performance. So, as with lactate, the explanation could be due to minority bacteria.

For butyrate-MECs, a difference of 56.81 % was observed between the most efficient reactor (Butyrate-4, 1.76 A.m⁻²) and the least efficient reactor (Butyrate-1, 1.00 A.m⁻²). In the *Geobacter* genus Butyrate-4 sample is composed of 74.58 % of *G. toluenoxydans* compared to

Butyrate-1 (0.33 %). Thus, *G. toluenoxydans* could be an effective species for the conversion of butyrate to current and this seems to be consistent with its ability to use this metabolite directly, unlike the other OTUs mainly present in other biofilms (*G. sulfurreducens* & *G. pelophilus*) [174].

2.3.3.5 Hypothetical distribution of electrons from substrates to different electron sinks

In order to better understand the reasons why a bacterial species can increase (effective species) or decrease (ineffective species) the MEC performance, it is interesting to study the different hypothetical pathways involved in metabolite degradation. Based on experimental electron distribution, community analysis and bibliographic knowledge, the electron flux from the substrates could pass through various routes [7, 17]. Each route involves specific microbial communities such as fermenters, EABs and syntrophic hydrogenotrophs (EABs). The first possible route involved fermentation step with respect to lactate in the formation of propionate and acetate in a 2:1 molar ratio [177]. No fermentative metabolites were detected during experiments with propionate- and butyrate-fed MECs suggesting direct conversion (path 2) into current by EABs such as *G. toluenoxydans*. The third (acetate/H₂) and four (formate) pathways are specific to syntrophic interactions. In the cases where they are not directly oxidized to current, the oxidation of fermentable substrates could produce intermediate metabolites which involved a microbial partnership between producers (acetate/H₂ or formate) such as *G. metallireducens* and consumers (i.e. EABs) such as *G. pelophilus* or *G. sulfurreducens* (Fig. 2-6). Thus, the oxidation of the previous substrates produces acetate/hydrogen (path 3) or formate (path 4) which can further be oxidized by syntrophic partners such as EABs to convert

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them into electricity [178]. A summary of the different possible routes can be seen in Schematic 2-1. The syntrophic pathway is less effective than the direct pathway, due to thermodynamic limitations to maintain low partial hydrogen pressure [68]. This is the reason why, depending on the species, the degradation pathways will be different (direct or indirect) which can impact the current densities. Experiments with synthetic bacterial consortia are necessary to validate these different hypotheses for a better understanding of the bacterial interactions.

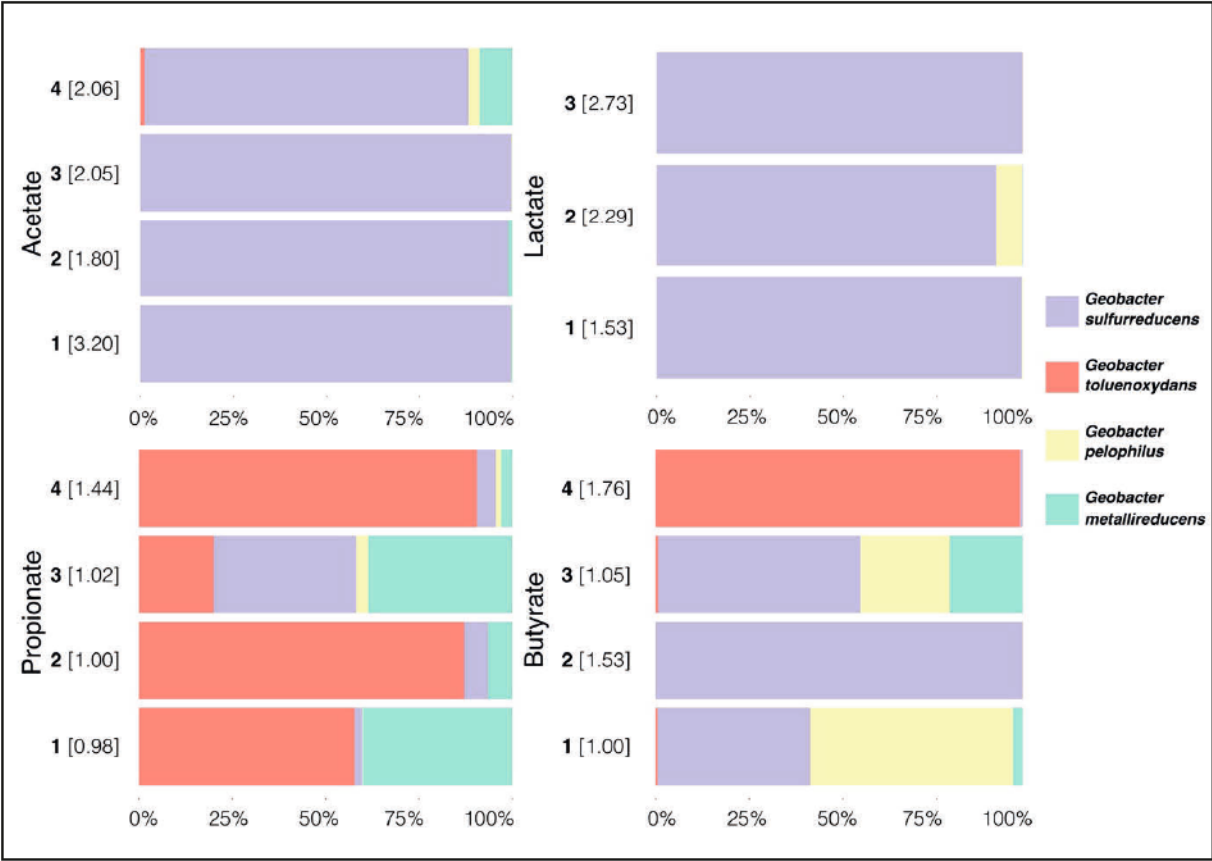
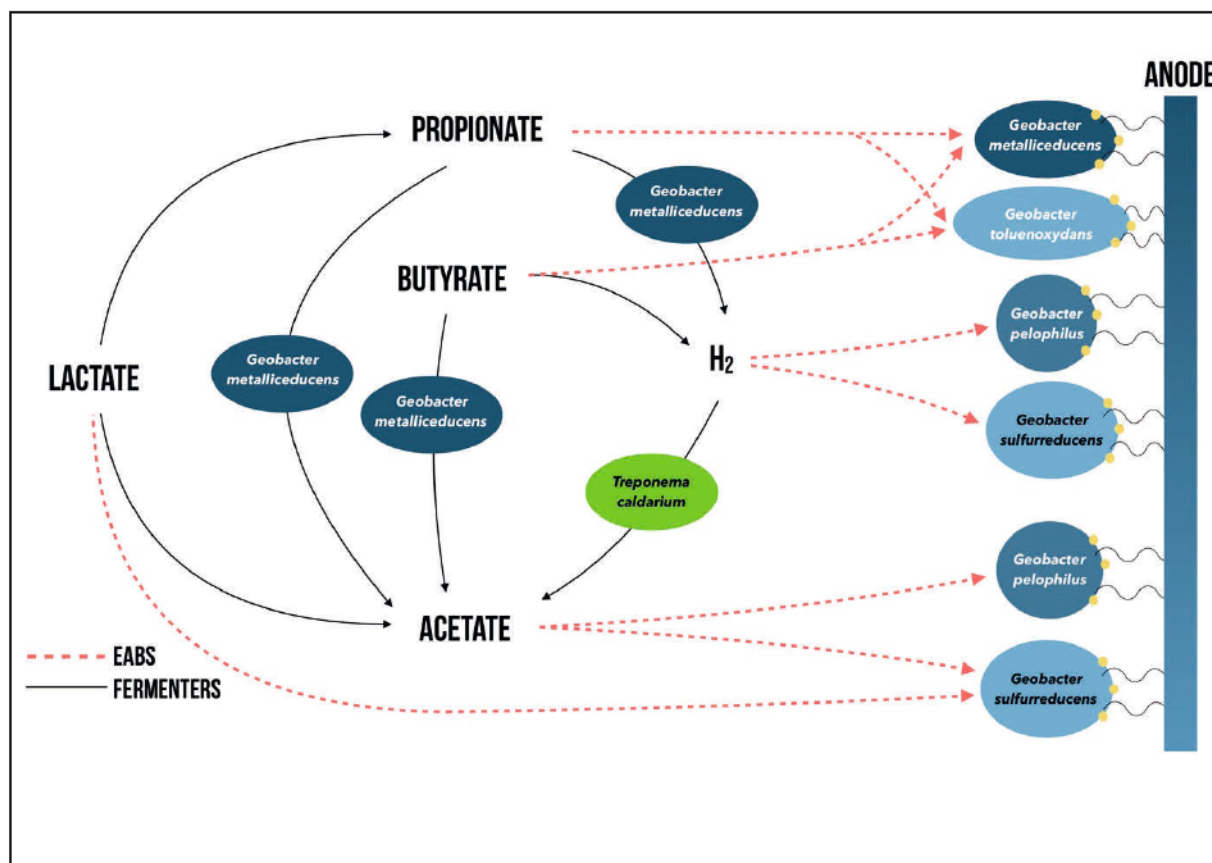


Fig. 2-6 Relative abundances of the main *Geobacter* species found in the anodic biofilms according to the fed substrate. The four *Geobacter* sp. represented 62.41% of total biofilm's sequences. Each *Geobacter* sp. is identified by one specific colour. Numbers (1-4) in bold are

specific to the replicate (except Lactate-MEC 4). Numbers in brackets correspond to the maximum current density ($A.m^{-2}$) of the sample.



Schematic 2-1 Hypothetical degradation pathways of the substrates tested in this study associated with anodic microorganisms mapping according to their metabolic potentials and abundances of community structures.

Conclusion

Lactate- and acetate-fed MECs showed higher performances in term of current densities and coulombic efficiencies with regard to those fed with butyrate and propionate. The biofilms diversity was the lowest when compared to bulks and inocula samples, indicating a selective

growth on anode as sole electron acceptor. Analysis of the microbial communities showed a predominance of the *Geobacteraceae* family (62.41% of the total sequences) but a different distribution at the *Geobacter* species-level according to the substrate. On the one hand *G. sulfurreducens* appears to be involved in competitive relationships in presence of acetate beside *G. metallireducens* and *G. pelophilus*. More complex substrates such as propionate and butyrate appear to induce syntrophic interactions between acetate producers (e.g. *G. metallireducens*) and consumers (e.g. *G. sulfurreducens* and *G. pelophilus*). Regarding the link between bacterial communities and performances, *Treponema caldarium* appears to be inefficient in the case of acetate oxidation, while *G. toluenoxydans* appears to be efficient for the conversion of propionate to current by its ability to use this metabolite directly without establishing syntrophy. Finally, these results allowing for the first time to make hypotheses of the ecological relationships existing within electroactive consortia as well as the ‘barrier effect’ that was probably caused by *G. sulfurreducens* and its low metabolic versatility preventing propionate oxidation. Consequently, it would be interesting to better understand the ‘barrier effect’ and the means to balance the electroactive ecosystems with propionate effective-species to promote propionate oxidation with acetate in solution.

Acknowledgements

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Ouverture

L'analyse des communautés microbiennes a montré une prédominance de la famille des *Geobacteraceae* (62,41% des séquences totales), mais une distribution différente au niveau de l'espèce selon le substrat. *Geobacter sulfurreducens* semble être impliquée dans des relations compétitrices en présence d'acétate à côté de *Geobacter metallireducens* et *Geobacter pelophilus*. Des substrats plus complexes comme le propionate et le butyrate semblent induire des interactions syntrophiques entre les producteurs d'acétate (par exemple *G. metallireducens*) et les consommateurs (par exemple *G. sulfurreducens* et *G. pelophilus*).

L'analyse du chapitre 2 s'est focalisée principalement sur le genre *Geobacter*. Cependant, les communautés électroactives sont composées de nombreuses autres espèces pouvant apporter des fonctions clés pour la communauté. Une analyse écologique complémentaire est indispensable pour comprendre la contribution d'autres taxa au processus d'oxydation des acides organiques en MECs. Par conséquent, les mêmes données ont été analysées avec un filtre d'abondance à 1% dans au moins un échantillon (contre 3% pour l'étude précédente). Un tel filtre permet la sélection de bactéries à plus faible abondance, mais augmente la complexité de l'analyse avec une augmentation de 14 (filtre 3%) à 22 OTUs (filtre 1%) concernant les biofilms et une augmentation de 22 (filtre à 3%) à 64 OTUs (filtre 1%) concernant les bactéries planctoniques. Pour réduire la complexité du système et se focaliser sur les bactéries d'intérêt, l'étude sous l'angle du core-microbiome, c'est à dire des bactéries en commun dans l'ensemble des échantillons (soit en mode biofilm, soit planctoniques) a été envisagée. Cette analyse avait pour objectif de caractériser le rôle des bactéries communes dans les biofilms et la partie planctonique quel

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que soit l'acide organique utilisé. Avec cette étude, quatre bactéries ont été sélectionnées dans les biofilms et huit dans le compartiment planctonique. Un réseau métabolique a été établi pour les deux consortia identifiés (anodique & planctonique). Ce travail est présenté en détail dans le chapitre suivant (Chapitre 3).

3 Chapter 3. A core-microbiome approach to identify key microbes and interactions in electroactive microbial communities

Avant-propos

Les consortia électroactifs sont des systèmes complexes et peu compris. Avec 2768 OTUs qui étaient présentes sous forme de biofilm et dans la partie planctonique, il était nécessaire pour identifier les espèces clés d'utiliser des méthodes analytiques réduisant la complexité. Dans cet objectif, l'analyse sous l'angle du core-microbiome, c'est à dire des bactéries communes à une niche écologique donnée (ici soit sous forme de biofilm, soit sous forme planctonique) a été mise en place dans l'objectif d'établir un réseau métabolique potentiel des consortia fermentaires et électroactifs. Cette étude propose une analyse en cinq étapes : (1) identification des core-OTUs ; (2) affiliation taxonomique et identification des métabolismes potentiels ; (3) corrélations entre les core-OTUs ; (4) corrélations entre les core-OTU et les indices de performance (c'est-à-dire CD_{max} et RF) et enfin (5) établissement d'un réseau métabolique potentiel. Cette étude propose un cadre analytique pour l'analyse des consortia microbiens complexes dans les systèmes bioélectrochimiques. Trois groupes fonctionnels ont été identifiés : les taxa fermentaires d'acides aminés, les hydrogénotrophes et les bactéries électroactives. Pour la première fois, il a été démontré une corrélation significative entre *Treponema caldarium*, une bactérie homoacétogène et les performances en termes de densités de courant maximales.

ABSTRACT

The microbial community structure of anodic biofilms and of planktonic communities plays a key role in bioelectrochemical systems. In these communities, the presence of numerous bacterial species having interconnected ecological interactions hinders the elucidation of their exact role in these systems. The objective of the present study was to identify key players in both biofilm and planktonic communities and to understand their interaction network.

Here 15 bioanodes were inoculated with the same activated sludge and fed with four different substrates: acetate, lactate, butyrate and propionate, at 80 mM- equivalents. Biofilm and planktonic communities were analyzed separately at the end of the batch operation. Key players of each community were then identified using a core-microbiome approach, i.e. analyzing microbes systematically found with all substrates and all replicates.

2768 OTUs were identified from the 30 microbial communities. 87 OTUs had abundance above 1% in at least one sample. Among them only 10 bacterial OTUs belonged to core-microbiomes. Core bacteria could be assigned to three putative functional groups: electro active bacteria, hydrogen scavengers and amino acids fermenters allowing drawing a map of putative metabolic interactions. The main electroactive species was *Geobacter sulfurreducens* which was present in both biofilm and planktonic core-microbiomes. Interestingly, one of the core biofilm OTUs was identified as a *Treponema caldarium* whose abundance was strongly negatively correlated with current density confirming that this genus could be used as a bioindicator of a loss of biofilm electroactivity.

Altogether this study illustrates how systematic replications and statistical approaches allow simplifying the study of complex microbial ecosystems through the identification of key players of the communities.

Keywords: Anodic core microbiome – Planktonic core microbiome – Ecological relationships – Bioanode – Microbial Electrolysis Cells

Introduction

Bioelectrochemical systems such as microbial electrolysis cells (MECs) or microbial fuel cells (MFCs) represent promising solutions for the production of renewable energy from organic waste [7]. These systems have in common the use of biological anodes (or bio-anodes) on which microorganisms grow via redox reactions using the anode as terminal electron acceptor [32]. Since the discovery of the potential to generate electricity from organic matter by Potter in 1911, numerous studies have more recently focused on the application of MXCs (i.e. MFC & MEC) for waste treatment to improve performance indices such as coulombic efficiency (%) or maximum production density ($A.m^{-2}$) with the aim of making this technology economically competitive for implementation in wastewater treatment plants [179, 180]. In the treatment of organic effluents in MXCs, a first fermentation step produces organic acids which must then be oxidized at the anode involving many bacterial species. However, the bacterial consortia and the ecological relationships present are poorly understood, especially because of the great bacterial diversity. Among these ecological relationships, the link between the

presence of hydrogen-scavengers, particularly homoacetogens, in electroactive biofilms and the performance variations observed in several bioelectrochemical systems studies remains unclear [7, 81, 95, 178, 181]. Indeed, in electroactive biofilms, hydrogen can be produced during fermentation and serve as electron donor for electroactive or homoacetogenic bacteria. There is therefore a need to better understand the trophic links between hydrogen producers and consumers to better understand these ecosystems [66]. New approaches are required to embrace this diversity in order to identify meaningful ecological patterns across electroactive consortia. The quest for core microbiomes analysis is a promising way [182]. The core-microbiome comprises common members shared by two or more microbial assemblages associated with a habitat [15]. Identifying the core species (or operational taxonomic units, OTUs) is essential to understand the ecology of microbial consortia because these commonly occurring microorganisms that appear in all assemblages associated with a particular habitat are likely essential to the function of that type of community [15]. The comprehension of the essential functions that microorganisms bring to the community is possible by a core-microbiome-analysis. This approach has been applied for example in acetate-fed microbial fuel cells and allowed the identification of *Geobacter* sp., *Aminiphilus* sp., *Sedimentibacter* sp., *Acetoanaerobium* sp., and *Spirochaeta* sp. in high power electroactive biofilms [16]. Thus, this analysis provides a better understanding of the ecological roles and interconnections of such electroactive consortia. Investigation of stochastic processes especially during the colonization of a new ecological niche (anode) from an inoculum with a high ecological diversity allows the characterization of bacteria well adapted to an ecological niche [183].

In this study, we analyzed the data of sixteen bioanodes fed with one of the four most common organic acids found in wastewater, i.e. acetate, butyrate, propionate and lactate [184]. Sequencing of microbial 16S ribotags from anolytes was performed as well as the analysis of the rest of the diversity, with the angle of the core-microbiome. A trophic network within planktonic and anodic consortia was carried out in five steps: (1) core-OTUs identification; (2) taxonomic affiliation and identification of potential metabolisms; (3) correlations among core-OTUs; (4) correlations between core-OTUs and performance indices (i.e. CD_{max} and CE) and finally (5) Establishment of a potential metabolic network. This study proposes an analytical framework for the analysis of complex microbial consortia in bio-electrochemical systems composed of three dominant functional groups: amino acids fermenters, H_2 -scavengers and electroactive bacteria.

3.1 Materials & Methods

The data from this experiment have already been used in a previous study [184]. The “Materials and Methods” is similar except for statistical analysis.

3.1.1 Statistical Analysis

All statistical analyses were carried out in R version 3.2.3 (R core Team 2014). The correlation tests were performed with the "Performance Analytics, v1.5.2" package. Venn diagrams were represented with the package "VennDiagram, v1.6.19" and “Phyloseq v1.22.3” [166]. The whole analytical methodology developed is detailed in "additional information" Fig. S1-2.

3.2 Results & Discussion

As mentioned in the ‘Materials and Methods’ section, the data used for the analysis of this paper were previously used in a previous study where *Geobacter* genus represented 62.4% of the total sequences of the biofilm samples [184]. These results indicated a specificity within the *Geobacter* genus towards the electron donor. At the species level, *Geobacter sulfurreducens* was dominant in presence of lactate and acetate, while *Geobacter toluenoxidans* and *Geobacter pelophilus* were dominant with butyrate and propionate as substrates. Overall, these results suggest a competitive process for electrode colonization and the implementations of syntrophic interactions for complete oxidation of substrates such as propionate and butyrate. Here, biofilm and bulk data were both considered in order to reconstitute a metabolic network with key species.

3.2.1 Core-OTUs analysis

A total of 2768 OTUs were identified from sequences obtained from bulk and biofilms. A first filter at 1% abundance in at least one sample was applied to select the OTUs that had a significant growth during at least one batch. This filter selected 23 and 64 OTUs for biofilm and bulk samples respectively. These pre-selected OTUs were used for the construction of Venn diagrams (Fig. 3-1.a & Fig. 3-2.a). At the centre of the diagram, the core-microbiome, composed of the core-OTUs (i.e. OTUs in common in either ‘biofilm’ or ‘bulk’) is represented

and in periphery the eco-microbiome, composed of the satellite-OTUs (i.e OTUs that were not systematically present in either ‘biofilms’ or ‘bulks’). The whole (core- & eco-microbiome), constitutes the pan-microbiome (for ‘biofilm’ or ‘bulk’ samples), composed of the pan-OTUs. The non-pan OTUs are composed of all the OTUs of the initial table minus the pan-OTUs in order to have an unbiased representation. The methodological procedure is available as additional information (Fig. S-1 & Fig. S-2).

The samples were composed of four bio-anodes and bulks sampled from microbial electrolysis cells (MECs) fed either with acetate (Acetate-1 to 4), butyrate (Butyrate-1 to 4), propionate (Propionate-1 to 4), and three lactate-fed bio-anodes and bulks (Lactate 1 to 3) as electron donors. Indeed, the 4th Lactate samples (Bio-anode & bulk) were not analyzed due to the poor quality of the reads.

3.2.2 Core-OTUs identification

3.2.2.1 Planktonic media

3.2.2.1.1 Planktonic microbiome

The planktonic pan-microbiome of filtered OTUs dataset is shown on the Venn diagram (Fig. 3-1.a). According to the Venn diagram, eight core-OTUs can be observed, fourteen OTUs into the eco-microbiome (satellite-OTUs) for a total of twenty-two OTUs constituting the planktonic pan-microbiome (Fig. 3-1.a).

The proportion of the three categories of OTUs, i.e. 'core', 'pan' and 'non-pan' can be observed Fig. 3-1.b. This graph shows the balance between non-pan OTUs (2746 OTUs) compared to the satellite-OTUs (14 OTUs) and the core-OTUs (8 OTUs). The planktonic core-microbiome was very selective with only 8 OTUs compared to the 2768 that made up the initial table. It can be observed that the cumulative abundances of core-OTUs was minor compared to non-pan OTUs on all planktonic samples. The core-OTUs was lowest in the Butyrate-4 sample (4.86%) and highest in the Acetate-2 sample (40.83%). However, the representativeness of the core-OTUs did not significantly differ between the substrates.

The relative composition of the core-OTUs can be observed in Fig. 3-1.c. The OTUs 3, 4 and 7 were respectively affiliated to *Macellibacteroides fermentans*, *Lutispora thermophila* and *Paraclostridium benzoelyticum* and were predominant whereas the OTU 25 represented by *Clostridium peptidivorans* had a low relative abundance in these consortia except for the Butyrate-4 sample where it was the second most abundant.

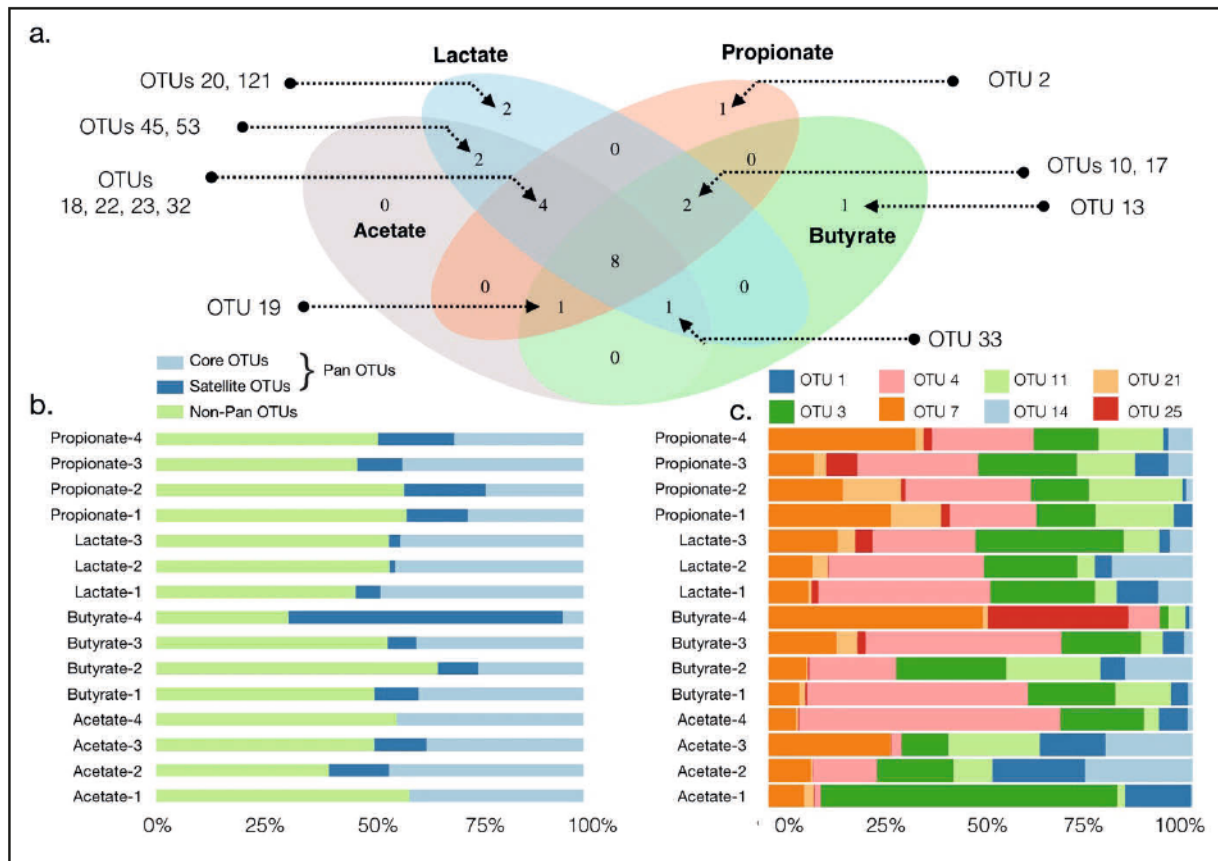


Fig. 3-1 a. Pan-microbiome of planktonic bacteria based on the filtered-OTUs selected from the entire OTUs dataset (At least 1% abundance in one). b. Representation of the distribution of non-pan OTUs compared to core- & satellite-OTUs in each sample. c. OTUs distribution within the planktonic core-microbiome associated with each sample. OTU 1 (*Geobacter sulfurreducens*), OTU 3 (*Macellibacteroides fermentans*), OTU 4 (*Lutispora thermophila*), OTU 7 (*Paraclostridium benzoelyticum*), OTU 11 (*Bilophila* sp.), OTU 14 (*Azonexus caeni*), OTU 21 (*Clostridium cylindrosporium*), OTU 25 (*Clostridium peptidivorans*).

3.2.2.1.2 Probable role of the planktonic core-OTUs

The potential metabolisms associated to each OTU can be observed in Table 3-1. The OTUs composing this core can be clustered into three hypothetical functional categories: (1) amino acids fermenters for OTU 3 (*Macellibacteroides fermentans*), OTU 4 (*Lutispora*

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thermophila), OTU 7 (*Paraclostridium benzoelyticum*), OTU 21 (*Clostridium cylindrosporum*) & OTU 25 (*Clostridium peptidivorans*); (2) H₂-scavenger for OTU 11 (*Bilophila* sp.) and (3) Electroactive for OTU 1 (*Geobacter sulfurreducens*) and OTU 14 (*Azonexus caeni*). Of the core-OTUs taxa, only OTU 11 was closed to a known hydrogen-scavenger, *Bilophila* sp. capable of oxidizing hydrogen and reducing CO₂ to produce acetate (homoacetogenic pathway) [185]. *G. sulfurreducens* is capable of using acetate, formate, lactate and H₂ as electron donors [60]. In planktonic mode, this EAB is capable of used self-secreted electronic shuttles, i.e. flavins, to use the anode as terminal electron acceptor at long range [186]. *A. caeni* is capable of using several electron donors such as acetate or propionate and an anode as terminal electron acceptor [187].

In the liquid medium, the only electron acceptors were amino acids provided by the yeast extract. These molecules could also be used to form biomass. Once they were consumed, no electron acceptor was present in solution. Table 3-2 presents the main fermentation reactions of amino acids and the variations of free enthalpy. It can be observed that the fermentations of alanine, leucine and glycine require a syntrophy with H₂-consuming organisms because of a positive free enthalpy variation compared to the other amino acids. It was possible to oxidize amino acids or organic molecules (acetate, lactate, propionate or butyrate) either by electronic transfer to the anode acting as terminal electron acceptor (i.e. H₂ or electronic shuttles such as flavins) or through the establishment of syntrophies with hydrogen consumers to make the reactions thermodynamically favorable. It would be interesting to know whether there were interdependence relationships between these H₂-scavengers and the other planktonic OTUs of the core-microbiome.

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In summary, the planktonic core-microbiome might be able to use (1) amino acids (OTU 3, 4, 7, 21 and 25); (2) hydrogen (OTUs 11) and (3) acetate (OTU 1 and 14) as substrates. Table 3-1 summarizes the hypothetical OTUs functions of the planktonic pan-microbiome.

Table 3-1 Summary table of the OTUs constituting the planktonic pan-microbiome of this study. For each OTU it is notified the percentage of identity with the reference (NCBI), the probable role, the substrates that it can use (For an exhaustive list it is possible to consult the associated references), the references used (Ref) and the electrons donor corresponding to the operational condition. OTUs 17, 19, 22, 53, 121 are unclassified.

Taxa	OTU n° [%id]	Probable role	Substrat(s)	Product(s)	Ref.	Electrons donor(s)
<i>Geobacter sulfurreducens</i>	1 [100]	?	Acetate, Formate, Lactate, H ₂	e ⁻ , H ₂ , CO ₂ Lactate,	[61]	All
<i>Macellibacteroides fermentans</i>	3 [100]	Yeast extract fermentation.	Yeast extract	Acetate, Butyrate, Isobutyrate	[188]	All
<i>Lutispora thermophila</i>	4 [96]	Amino acids fermentation	Peptone, Tryptone, Casamino acids, Casein hydrolysate, Methionine, Tryptophan, Tryptophan, Cysteine, Lysine, Serine, Yeast extract	Acetate, Iso- butyrate, Propionate, Iso- valerate	Shirator et al, 2008 [189]	All
<i>Paraclostridium benzoelyticum</i>	7 [100]	Amino acids fermentation	Amino acids	H ₂ , CO ₂	[190]	All

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<i>Bilophila</i> sp.	11 [95]	H ₂ -scavenger	H ₂ + CO ₂	Acetate	[185]	All
			acetate, malate, propionate, valerate, 3- hydroxybutyrate, l- proline, dl-lactate and malonate	e ⁻ , H ⁺ , CO ₂	[187]	All
<i>Azonexus caeni</i>	14 [100]	?	Glycine, Uric acid, Xanthine, Guanine, 6,8- dihydroxypurine, hypoxanthine	Acetate, Formate, CO ₂ , NH ₄ ⁺	[191]	All
<i>Clostridium cylindrosporum</i>	21 [95]	Amino acids fermentation	Yeast extract, Casamino acids, gelatin, peptone, arginine, lysine, cysteine, methionine, histidine, serine, isoleucine and crotonate	Acetate, H ₂ , CO ₂ , Propionate, Butyrate, Ethanol, Alanine, methyl 2-butyrate	[192]	All
<i>Clostridium peptidivorans</i>	25 [100]	Amino acids fermentation	Acetate, Lactate, Succinate	e ⁻ , H ⁺ , CO ₂	[127]	Acetate, Lactate, Butyrate
<i>Arcobacter butzleri</i>	33 [98]	?	Acetate, Lysine, Arginine, D-Glucose, many sugars and polyhydroxyl acohols	H ₂ , CO ₂	[193]	Acetate, Lactate, Propionate

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<i>Aeromonas hydrophila</i>	23 [100]	?	Acetate	e^- , H^+ , CO_2	[194]	Acetate, Lactate, Propionate
<i>Citrobacter</i> sp.	32 [99]	?	Acetate, Citrate, Glucose, Sucrose, Glycerol, Lactose	e^- , H^+ , CO_2	[195]	Acetate, Lactate, Propionate
<i>Azovibrio restrictus</i>	10 [100]	Amino acids fermentation	Propionate, L-aspartate	H_2 , CO_2	[196]	Lactate, Propionate, Butyrate
<i>Proteocatella sphenisci</i>	45 [100]	Amino acids fermentation	Yeast extract, amino acids, casamino acids, peptone	Acetate, Butyrate, Ethanol, H_2	[197]	Lactate, Acetate
<i>Clostridium ganghwense</i>	20 [98]	?	Glucose, Maltose, Salicine, Cellobiose, Mannose	Glycerol, Ethanol, CO_2	[198]	Lactate
<i>Geobacter toluenoxidans</i>	2 [97]	?	Acetate, Propionate, Butyrate	e^- , H_2 , CO_2	[174]	Propionate
<i>Dechloromonas</i> sp.	13 [95]	?	Acetate, Propionate	Cl_2	[199]	Butyrate

Table 3-2 Changes of Gibbs free energies under standard conditions in hydrogen-releasing reactions during fermentation of amino acids. a. These fermentations may also allow growth in pure culture. b. These reactions are always coupled to further fermentation of glutamate. All calculations are based on published tables [200].

Reactants	Products	$\Delta G_o'$ (kJ per mol rect.)
Alanine + $2H_2O$	acetate ⁻ + CO_2 + NH_4^+ + $2H_2$	+2.7
Leucine + $3H_2O$	acetate ⁻ + HCO_3^- + H^+ + NH_4^+ + $2H_2$	+7.5

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Glycine + 2H ₂ O + H ⁺	2CO ₂ + NH ₄ ⁺ + 2H ₂	+17.8
Serine + H ₂ O	acetate ⁻ + CO ₂ + NH ₄ ⁺ + H ₂	-85.3 ^a
Threonine + H ₂ O	propionate ⁻ + CO ₂ + NH ₄ ⁺ + H ₂	-83.0 ^a
Histidine + 4H ₂ O + H ⁺	glutamate ⁻ + CO ₂ + 2NH ₄ ⁺ + H ₂	b
Proline + 2 H ₂ O	glutamate ⁻ + H ⁺ + 2H ₂	b
Glutamate ⁻ + 2H ₂ O + H ⁺	propionate ⁻ + 2CO ₂ + NH ₄ ⁺ + 2H ₂	-16.6
Glutamate ⁻ + 2H ₂ O	2 acetate ⁻ + CO ₂ + NH ₄ ⁺ + H ₂	-38.6 ^a
Aspartate ⁻ + 2H ₂ O + H ⁺	acetate ⁻ + 2CO ₂ + NH ₄ ⁺ + 2H ₂	-24.1

3.2.2.2 *Electroactive biofilms*

3.2.2.2.1 *Anodic microbiome*

The anodic pan-microbiome of filtered OTUs dataset is shown on the Venn diagram (Fig. 3-2.a). According to the Venn diagram, four OTUs (core-OTUs) can be observed in the core-microbiome, nineteen OTUs into the eco-microbiome (satellite-OTUs) for a total of twenty-three OTUs constituting the pan-microbiome (Fig. 3-2.a).

The proportion of the three categories of OTUs, i.e. 'core', 'pan' and 'non-pan' can be observed in Fig. 3-2.b. This graph shows the balance between non-pan OTUs (2745 OTUs) compared to the satellite-OTUs (19 OTUs) and the core-OTUs (4 OTUs). The cumulative abundances of core-OTUs was predominant for bio-anodes fed with acetate 73.28 \pm 11.17 % and lactate 73.67 \pm 2.36% with a maximum for the Acetate-3 (83.67%) sample. However, the latter was smaller in three of the four bio-anodes fed with butyrate with a mean of 39.35 \pm 28.23% with Butyrate-1, -3 and -4 and only 3.2% on the sample Butyrate-2 sample. Concerning propionate-fed bio-anodes, all had a minority core-microbiome with an average of 20.10 \pm 10.6%.

The relative composition of the core-OTUs can be observed Fig. 3-2.c. OTU 1 was close to *G. sulfurreducens* and dominated all samples except for the Propionate-1-2 & 4 samples dominated by OTU 9 close to Bacteroidetes with an abundance of 55.01, 48.74 and 44.04% respectively. The biofilm core-microbiome represented a very small number of OTUs ($n = 4$) which indicates a very high selectivity with respect to the 2768 OTUs in total. Despite their small number, the core-OTUs were predominant on more than half of the bio-anodes, meaning a critical ecological role for the community [15]. Interestingly, core-OTUs were in the minority on half of the butyrate-fed bio-anodes and all the propionate-fed bio-anodes (Fig. 3-2.c). Therefore, it seems to be a relationship between the substrate and the functional importance of core-OTUs. It appears that the more complex the substrate (propionate and butyrate) the more microbial diversity is associated. If the relative abundance of this core-microbiome decreases, it means that its associated ecological functions were not sufficient for an operational ecosystem. Interestingly, it can be observed that OTUs 1 & 7 were present in the both core-

microbiomes (planktonic & biofilm). To understand the core functionalities, it is necessary to analyze the probable role of these OTUs (Fig. 3-2.c).

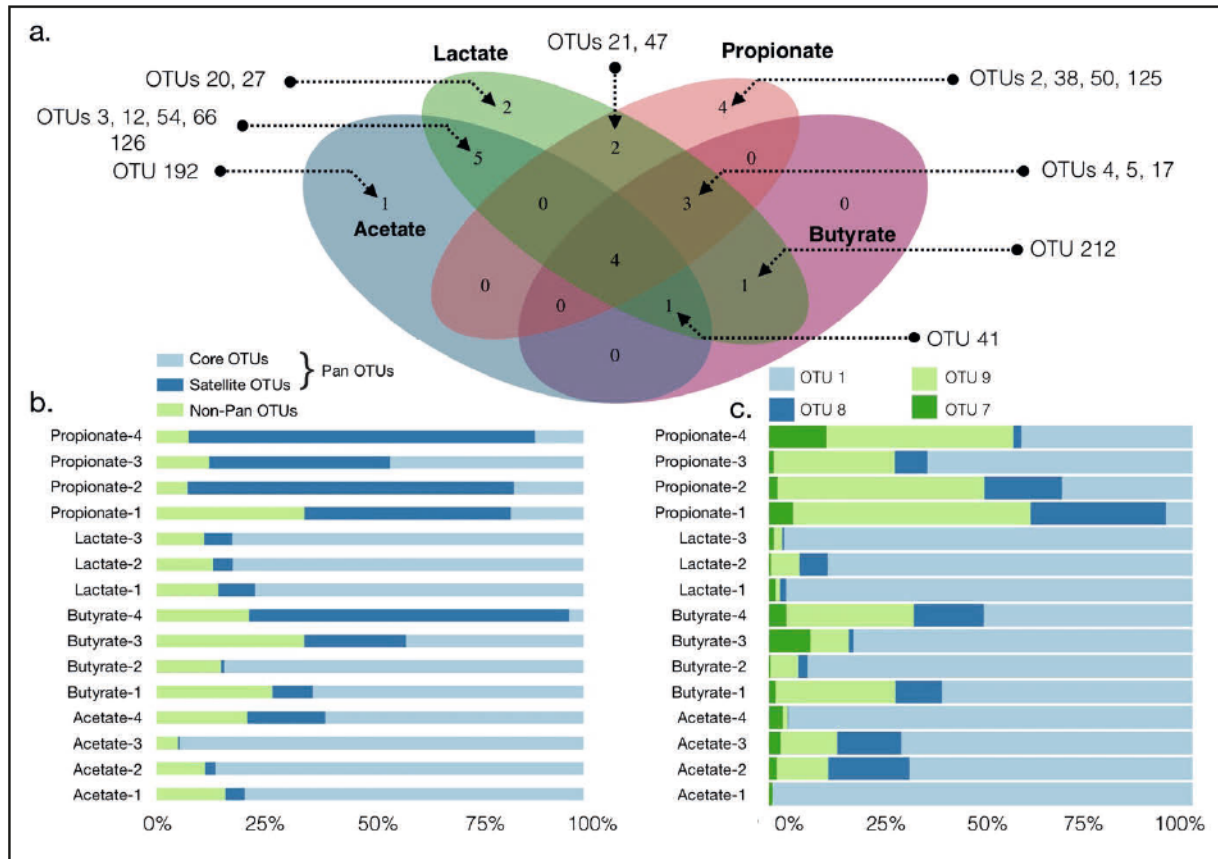


Fig. 3-2 a. Pan-microbiome of bio-anodes based on the filtered-OTUs selected from the entire OTUs dataset (At least 1% abundance in one sample). b. Representation of the distribution of non-an OTUs compared to core- & satellite-OTUs in each sample. c. OTUs distribution within the biofilm core-microbiome associated with each sample. OTU 1 (*Geobacter sulfurreducens*), OTU 7 (*Paraclostridium benzoelyticum*), OTU 8 (*Treponema caldarium*), OTU 9 (Unclassified).

3.2.2.2.2 Probable role of the biofilm core-OTUs

The potential metabolisms associated to each OTU can be observed Table 3-3. The OTUs composing this core can be clustered into three hypothetical functional categories: (1) amino acids fermenter for OTU 7 (*Paraclostridium benzoelyticum*); (2) H₂-scavenger for OTU 8 (*T. caldarium*) and (3) Electroactive for OTU 1 (*G. sulfurreducens*). OTU 9 was not classifiable at the genus level. Its function remains unknown.

The most represented OTU was closely related to *Geobacter sulfurreducens* (OTU 1). It is known as electro-active, i.e. capable of using the anode as a terminal electron acceptor [60, 72]. In the absence of any electron acceptor, *G. sulfurreducens* is able to use the redox active protein (e.g. c-type cytochromes) of other fermentative bacteria to carry out its respiratory process [56]. *G. sulfurreducens* is capable of using acetate, formate, lactate and H₂ as electron donors. In the core-microbiome, this OTU could give the ability to use the anode as terminal electron acceptor, as well as acetate present in the growing media of the MECs fed with acetate and lactate (the lactate is rapidly fermented in 2: propionate 1: acetate, see '[184]') as electron donor. However, this EAB is unable of using propionate and butyrate, which may explain its lower proportion among biofilm bacteria fed with these two substrates as electron-donors, suggesting the presence of another electroactive species in the eco-microbiome. The OTU 9 was closed to Bacteroidetes taxa. It is not possible to understand its probable role within this consortium due to this low taxonomic resolution (i.e. Phylum). OTU 7 was closely related to *Paraclostridium benzoelyticum*. This taxon is able to ferment amino acids and produce H₂ and CO₂ [190]. Surprisingly, only one known amino acid fermenter was present in the core-microbiome. The OTU 8 is represented by *Treponema caldarium*, capable of using H₂ and CO₂ to produce acetate via the homo-acetogenesis pathway [176, 201].

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Table 3-3 Summary table of the OTUs constituting the biofilm pan-microbiome of this study. For each OTU it is notified the percentage of identity with the reference (NCBI), the probable role, the substrates that it can use (For an exhaustive list it is possible to consult the associated references), the references used (Ref) and the electrons donor with which it is present. OTUs 9, 17, 21, 38, 50, 54, 66, 125, 192, 212 are unclassified.

Taxa	OTU n° [%id]	Probable role	Substrat(s)	Product(s)	Ref.	Electrons donor(s)
<i>Geobacter sulfurreducens</i>	1 [100]	Anode respiration	Acetate, Formate, Lactate, H ₂	e ⁻ , H ₂ , CO ₂	[61]	All
<i>Paraclostridium benzoelyticum</i>	7 [100]	Amino acids fermentation	Amino acids	H ₂ , CO ₂	[190]	All
<i>Treponema caldarium</i>	8 [99]	H ₂ -scavenger	H ₂ , CO ₂	Acetate	[202]	All
<i>Geobacter metallireducens</i>	5 [100]	Anode respiration	Acetate, Propionate, Butyrate Peptone, Tryptone, Casamino acids, Casein	e ⁻ , H ₂ , CO ₂	[203]	Lactate, Butyrate, Propionate
<i>Lutispora thermophila</i>	4 [96]	Amino acids fermentation	hydrolysate, Methionine, Tryptophan, Tryptophan, Cysteine, Lysine, Serine, Yeast extract	Acetate, Iso- butyrate, Propionate, Iso-valerate	Shirator et al, 2008 [189]	Lactate, Butyrate, Propionate
<i>Seleniivibrio woodruffii</i>	41 [100]	?	Acetate	?	[168]	Acetate, Butyrate, Lactate
<i>Geobacter metallireducens</i>	47 [98]	Anode respiration	Acetate, Propionate, Butyrate	e ⁻ , H ₂ , CO ₂	[203]	Lactate, Propionate

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				Lactate,		
<i>Macellibacteroides fermentans</i>	3 [100]	Yeast extract fermentation.	Yeast extract	Acetate, Butyrate, Isobutyrate	[188]	Acetate, Lactate
<i>Geobacter pelophilus</i>	12 [97]	Anode respiration	H ₂ , Formate, Acetate, Pyruvate, Succinate, Fumarate	e ⁻ , H ⁺ , CO ₂	[172]	Acetate, Lactate
<i>Clostridium acidisoli</i>	126 [95]	?	Glucose, cellobiose, xylose, arabinose, maltose, mannose, salicin, mannitol, lactose, sucrose, glycerol, melezitose, raffinose, rhamnose	Acetate, Butyrate, Lactate, H ₂ , CO ₂	[204]	Acetate, Lactate
<i>Clostridium cylindrosporium</i>	21 [95]	?	Glycine, Uric acid, Xanthine, Guanine, 6,8-dihydroxypurine, hypoxanthine	Acetate, Formate, CO ₂ , NH ₄ ⁺	[191]	Lactate
<i>Geobacter metallireducens</i>	27 [98]	Anode respiration	Acetate, Propionate, Butyrate	e ⁻ , H ₂ , CO ₂	[203]	Lactate
<i>Geobacter toluenoxidans</i>	2 [97]	Anode respiration	Acetate, Propionate, Butyrate	e ⁻ , H ₂ , CO ₂	[174]	Propionate

3.2.2.2.3 Relationship between amino acid fermenters and H₂-scavengers

To better understand the relationship between amino acid fermenters and hydrogen-scavengers in electroactive biofilms, the changes of Gibbs free energies during the fermentation step can be observed in Table 3-2.

Hydrogen production results from the fermentation of the main amino acids into organic acids. Among them, three reactions are not thermodynamically favorable under standard conditions: alanine, leucine and glycine fermentation with a $\Delta G^{\circ\prime} > 0$. In addition, the conversion of glutamate to propionate and aspartate to acetate is close to 0 with -16.6 and -24.1 kJ/mole respectively. Therefore, to ferment certain amino acids such as alanine and glycine, a syntrophic interaction is necessary. In methanogenic environments, methanogens play the role of H₂-scavengers. Without methanogens, the degradation of alanine, valine and isoleucine can be hindered [205]. Nanninga and Gottschal (1985) demonstrated stimulation of amino acid degradation when hydrogen scavengers were added [206]. So, it would appear that the same type of interaction could take place within electroactive biofilms in a very selective way. However, the presence of a single amino acid fermenter in the biofilm core-microbiome remains unclear.

To understand whether other amino acid fermenters and H₂-scavengers (EABs or homoacetogenic bacteria) were present in other electroactive biofilms fed with one electron source, a summary of other publications is presented in Table 3-4. It can first be observed that no amino acid fermenter has been detected when non-hydrogenotrophic EABs were present. On the contrary, when hydrogenotrophic-EABs were present, amino acid fermenters were also present, except for the study of [66]. This co-occurrence seems to support the hypothesis that amino acid fermenters were favored by the presence of hydrogenotrophic-EABs which would make the fermentation of certain amino acids favorable. Surprisingly, few homoacetogens have been found. Only *Acetobacterium* sp. was detected on an ethanol-fed bioanode. Its presence, as

well as that of an ethanol fermenter (*Pelobacter* sp.) and an EAB (*Geobacter* sp.), revealed a three-way syntrophy for the conversion of ethanol into electrons.

It may be interesting to understand how, in a highly selective consortium, two H₂-scavengers can co-exist. This fact has already been observed on a bio-anode, during a co-culture between *G. sulfurreducens* and *Hydrogenophaga*, both hydrogenotrophic electroactive bacteria [66]. Acetate was the only electron donor while *Hydrogenophaga* could not use it. To explain his presence, this study showed that when *G. sulfurreducens* is not in contact with any electron acceptor (Fe(III) or anode), the latter can oxidize acetate only at low hydrogen partial pressure (150 Pa) due to syntrophy with an hydrogen consumer. Therefore, in our study, it is possible that *T. caldarium* may be related to *G. sulfurreducens* cells in the biofilm that were not in contact with the anode. This could explain its systematic co-occurrence in all biofilms with *G. sulfurreducens*.

In summary, the biofilm core-microbiome appears capable of using (1) amino acids (OTU 9); (2) hydrogen (OTUs 8 & 1); (3) acetate (OTU 1) as substrates and (4) anode (OTU 1) as a terminal electron acceptor. These four functions were therefore intrinsic to this core-microbiome and distributed throughout all the bio-anodes of this study. Table 3-3 summarizes the hypothetical OTUs functions of the biofilm pan-microbiome.

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Table 3-4 Summary of the different taxa found in other studies using bioelectrochemical systems with a single electron donor substrate.

Substrats	Non-hydrogenotrophic EABs	hydrogenotrophic EABs	Amino acids fermenters	Homo-acetogenic bacteria	Ref.
	<i>Shewanella</i> sp., <i>Pseudomonadaceae</i>	<i>Geobacter</i> sp., <i>Desulfovibrio</i> sp.	<i>Coprothermobacter</i> sp., <i>Synergistaceae</i> , <i>Dethiosulfovibrionaceae</i>	-	[207]
	-	<i>Geobacter</i> sp.	<i>Clostridium sticklandii</i> , <i>Clostridium aminobutyricum</i>	-	[67]
	<i>Geobacter psychrophilus</i> , <i>Pseudomonas</i> sp.	<i>Geobacter sulfurreducens</i> , <i>Desulfovibrio</i> sp.	<i>Proteiniphilum</i> sp.	-	[208]
	<i>Rhosopseudomonas palustris</i>	-	-	-	[73]
Acetate	-	<i>Hydrogenophaga</i> sp., <i>Geobacter sulfurreducens</i>	-	-	[66]
	-	<i>Geobacter sulfurreducens</i>	<i>Clostridium sticklandii</i>	-	[11]
	-	<i>Geobacter</i> sp.	<i>Sedimentibacter</i> , <i>Amiiphilus</i> , <i>Cloacibacillus</i>	-	[16]

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	-	<i>Geobacter</i> sp.	<i>Acidaminococaceae</i>	-	[74]
	-	<i>Geobacter</i> sp., <i>Desulfovibrio</i> sp.	<i>Sedimentibacter</i> sp., <i>Cloacibacillus</i> sp.	-	[17]
Propionate	<i>Shewanella</i> sp., <i>Pseudomonadaceae</i>	<i>Geobacter</i> sp., <i>Desulfovibrio</i> sp.	<i>Coprothermobacter</i> , <i>Synergistaceae</i> , <i>Dethiosulfovibrionaceae</i>	-	[207]
Lactate	<i>Shewanella</i> sp.	-	-	-	[209]
Ethanol	-	<i>Geobacter</i> sp., <i>Desulfovibrio</i> sp.	<i>Acetobacterium</i> sp.	-	[95]

3.2.3 Correlation among core-OTUs

3.2.3.1 Correlation analysis among planktonic core-OTUs

In Fig. 3-3, a correlation network among the planktonic-core OTUs absolute abundances can be observed. Among them, only five were significantly correlated with the same number ($n = 4$) of correlations links (symbolized by lines between OTUs in Fig. 3-3) indicating a strong OTU interdependence. These five OTUs include three amino acid recyclers (OTU 3 - *Mecellibacteroides fermentans*; OTU 4 - *Lutispora thermophila*; and OTU 7 - *Paraclostridium benzoelyticum*), an hydrogen-scavenger (OTU 11 - *Bilophila*) and an electroactive bacterium (OTU 1 - *G. sulfurreducens*). Acetate is a potential fermentation product from amino acids (Table 3-2). Therefore, *G. sulfurreducens* probably used this organic

acid as electron donor, which could explain these highly significant links. The terminal electron acceptor of this EAB was not characterized in the medium. Two solutions may exist in the light of the current knowledge. Either *G. sulfurreducens* could use self-secreted flavins as electronic shuttles to transfer its electrons towards the anode or this EAB could use the extracellular redox active proteins of amino acid fermenters as final electron acceptors [56, 186]. These fermentation pathways can also produce hydrogen, which would explain the co-occurrence of *Bilophila* sp. with amino acid fermenters. This latter might reduce the hydrogen partial pressure to make negative the free enthalpy and promote amino acids fermentation (Table 3-2) [185].

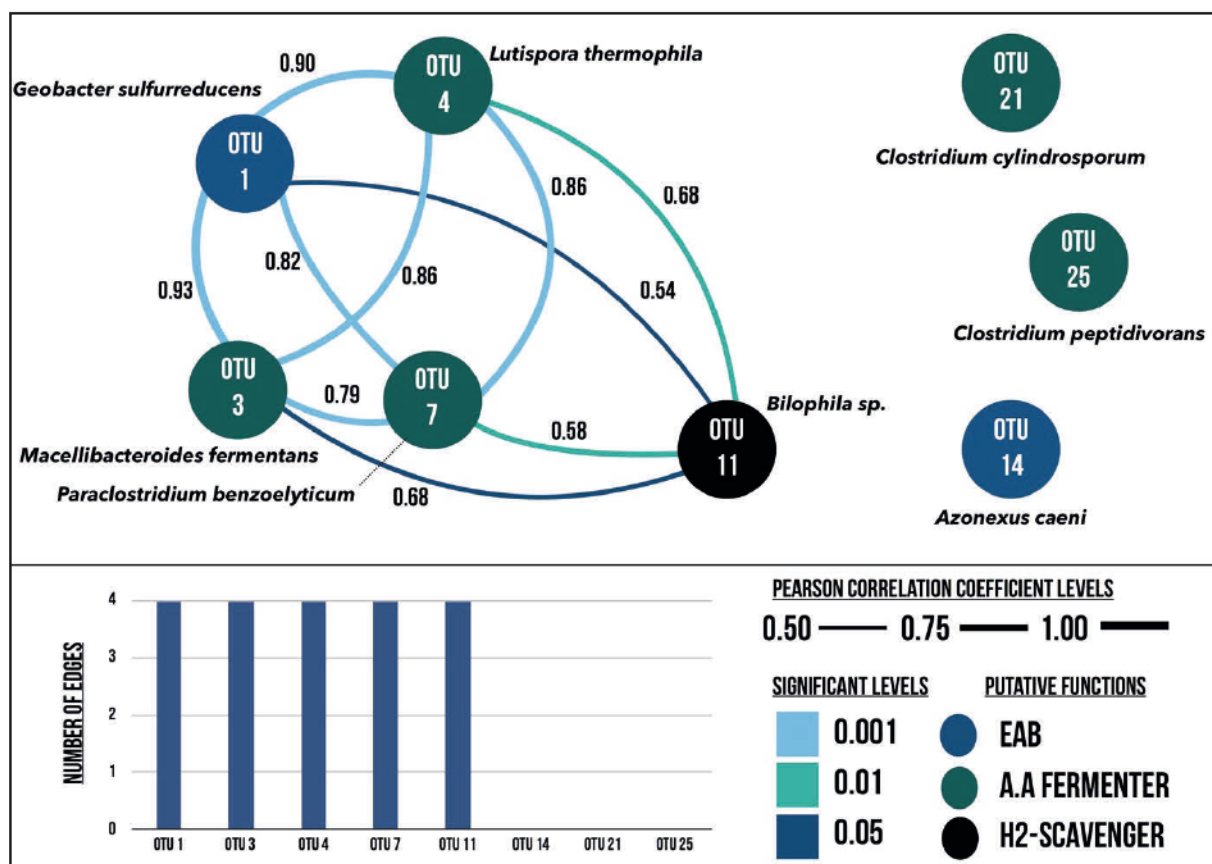


Fig. 3-3 Correlation network performed at the OTU level between planktonic core-OTUs. The line' sizes connecting two OTUs is relative to the *Pearson* coefficient. The detailed correlation diagram is available Fig. S-4 in supplementary material.

3.2.3.2 Correlations analysis among biofilm core- OTUs

To understand if there were relationships among the biofilm core-OTUs, a correlation network can be observed in Fig. 3-4. A significant correlation between OTU 9 and *T. caldarium* (OTU 8) can be seen ($r=0.85$). However, without knowing the OTU 9 metabolism, it is not possible to understand this significant interdependence. *P. benzoelyticum* (OTU 7) was

associated with *T. caldarium* with a *Pearson* coefficient of 0.66. The first is capable of producing H₂ and CO₂ from amino acids which were both substrates for *T. caldarium* [202]. Thus, these bacteria have a probably syntrophic relationship, in which *T. caldarium* acts as an hydrogen-scavenger based on its metabolic capacities. This OTU was also significantly correlated with *G. sulfurreducens* (OTU 1) with a *Pearson* coefficient of 0.59. As previously described, *G. sulfurreducens* is capable of oxidizing hydrogen directly to the anode [30]. Therefore, *P. benzoelyticum* would be in contact with two hydrogen consumers, OTU 1 & 8. *T. caldarium* can produce acetate via homo-acetogenesis pathway that can be used rapidly to the anode by *G. sulfurreducens*, which could explain their presence in the same core-microbiome even if the abundances of these two OTUs were not correlated.

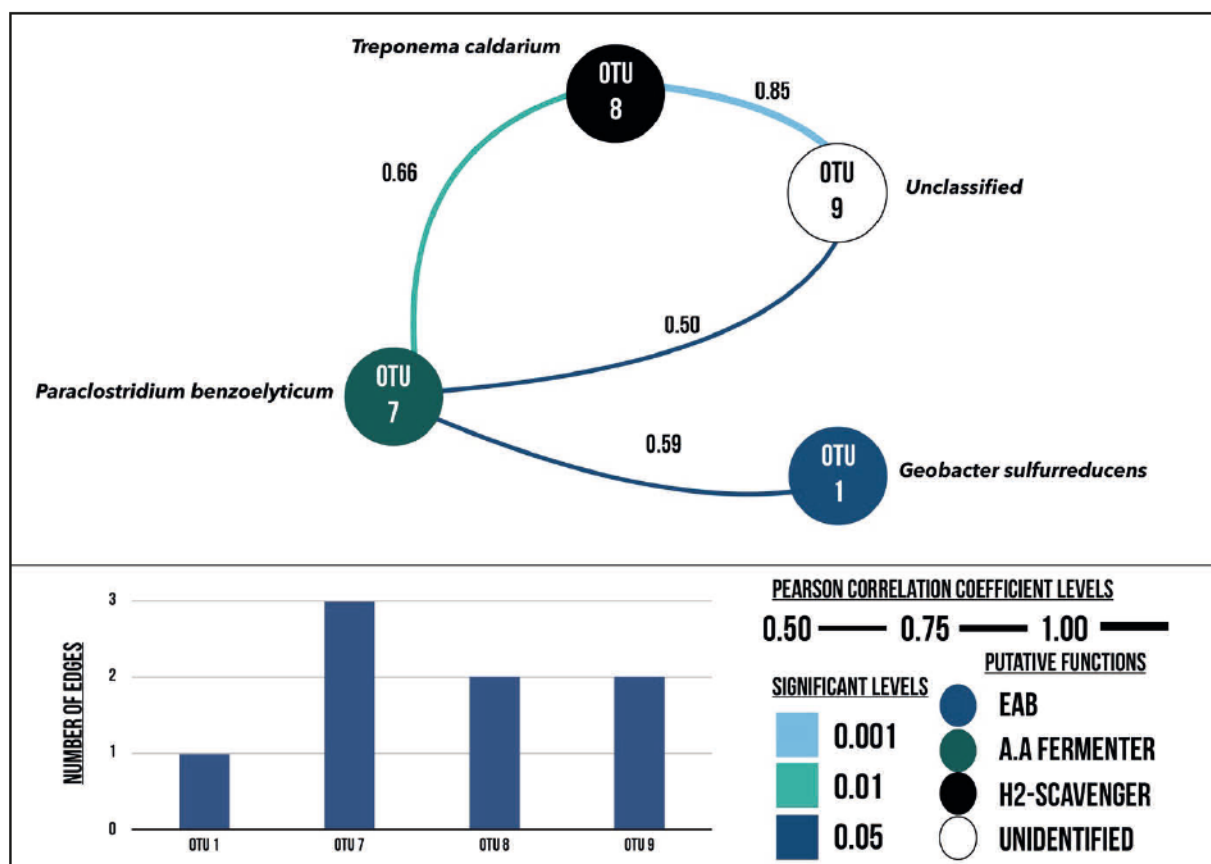


Fig. 3-4 Correlation network performed at the OTU level between anodic core-OTUs. The line' sizes connecting two OTUs is relative to the *Pearson* coefficient. The detailed correlation diagram is available Fig. S-5 in supplementary material.

3.2.4 Correlation analysis between core-OTUs and performance indices

3.2.4.1 Correlation analysis between planktonic core-OTUs and performance indices

To identify among the OTUs of the planktonic core-microbiome those correlated to CD_{max} and CE performance indices, a correlation analysis based on absolute abundances and

the *Pearson* index was performed. However, no significant relationship was observed (Table 3-5).

Table 3-5 Correlations (*Pearson* coefficient) between the absolute abundance of planktonic core-OTUs with the current density ($A.m^{-2}$) and the coulombic efficiency (%). OTU 1 (*Geobacter sulfurreducens*), OTU 3 (*Macellibacteroides fermentans*), OTU 4 (*Lutispora thermophila*) OTU 7 (*Paraclostridium benzoelyticum*), OTU 11 (*Bilophila* sp.), OTU 14 (*Azonexus caeni*), OTU 21 (*Clostridium cylindrosporium*), OTU 25 (*Clostridium peptidivorans*).

Performances indices	OTU 1	OTU 3	OTU 4	OTU 7	OTU 11	OTU 14	OTU 21	OTU 25
Current Density ($A.m^{-2}$)	.01	.24	.18	-.05	-.12	-.45	-.31	-.35
Coulombic efficiency (%)	.46	.38	.23	.3	.02	.14	.24	.12

3.2.4.2 Correlation analysis between anodic core-OTUs and performance indices

To identify OTUs of the anodic core-microbiome correlated to the CD_{max} and CE performance indices, a correlation analysis based on absolute abundances and the *Pearson* index was performed (Table 3-6). A significant inversely correlated link between the current densities and the abundances of *T. caldarium* was observed ($r = -0.95$, $p = 2.2 \cdot 10^{-8}$). Similar observations were previously reported for EAB from several bioelectrochemical systems studies [7, 81, 95, 178, 181]. To explain this correlation, *Treponema caldarium* (OTU 8) could

be related to the cells of *Geobacter sulfurreducens* (OTU 1) which were in the biofilm but not in direct contact with the anode. Indeed, when *G. sulfurreducens* does not have direct contact with the anode, it is capable of oxidizing acetate to H₂ and CO₂ with an hydrogenotrophic bacteria which decreases the hydrogen partial pressure and makes the reaction favorable [66]. The interaction between these two bacteria could also be through direct electron transfer already observed between *Geobacter sulfurreducens* and a syntrophic partner such as *Geobacter metallireducens* [210]. This syntrophic relation could explain that current densities have decreased due to the homoacetogenic pathway which involves a three-step path: (1) acetate to H₂ and CO₂; (2) H₂ and CO₂ to acetate; (3) acetate oxidation to the anode. This correlation supports the hypothesis that the presence of *T. caldarium* is strongly linked with current densities and could be linked with the *Geobacter sulfurreducens* cells which were not in direct contact with the anode. OTU 7 and 9 were inversely correlated to current density with $r = -0.74$ and $r = -0.79$ respectively. Despite these significant relationships, these links remain unclear.

OTU 1 & 7 were positively correlated to the coulombic efficiency with respectively $r=0.67$ and $r=0.57$ (Table 3-6). OTU 1 seems capable of using the main fermentation products (H₂ and acetate) directly at the anode, which could explain this correlation. On the other hand, OTU 7 which could be involved in the recycling of amino acids to H₂ could improve the efficiency of electron recovery to the anode (e.g. by recycling amino acids from dead cells [16]).

Table 3-6 Correlations (*Pearson* coefficient) between the absolute abundances of biofilm core-OTUs with the current density (A.m⁻²) and the coulombic efficiency (%) normalized. Significant correlations are indicated by an asterisk. The more asterisks, the more significant relationship is; * p value < .05, ** p value < .01, *** p value < .001). OTU 1 (*Geobacter*

sulfurreducens), OTU 7 (*Paraclostridium benzoelyticum*), OTU 8 (*Treponema caldarium*), OTU 9 (Unclassified). Fig.S 5-9 show significant correlations in scatterplot form.

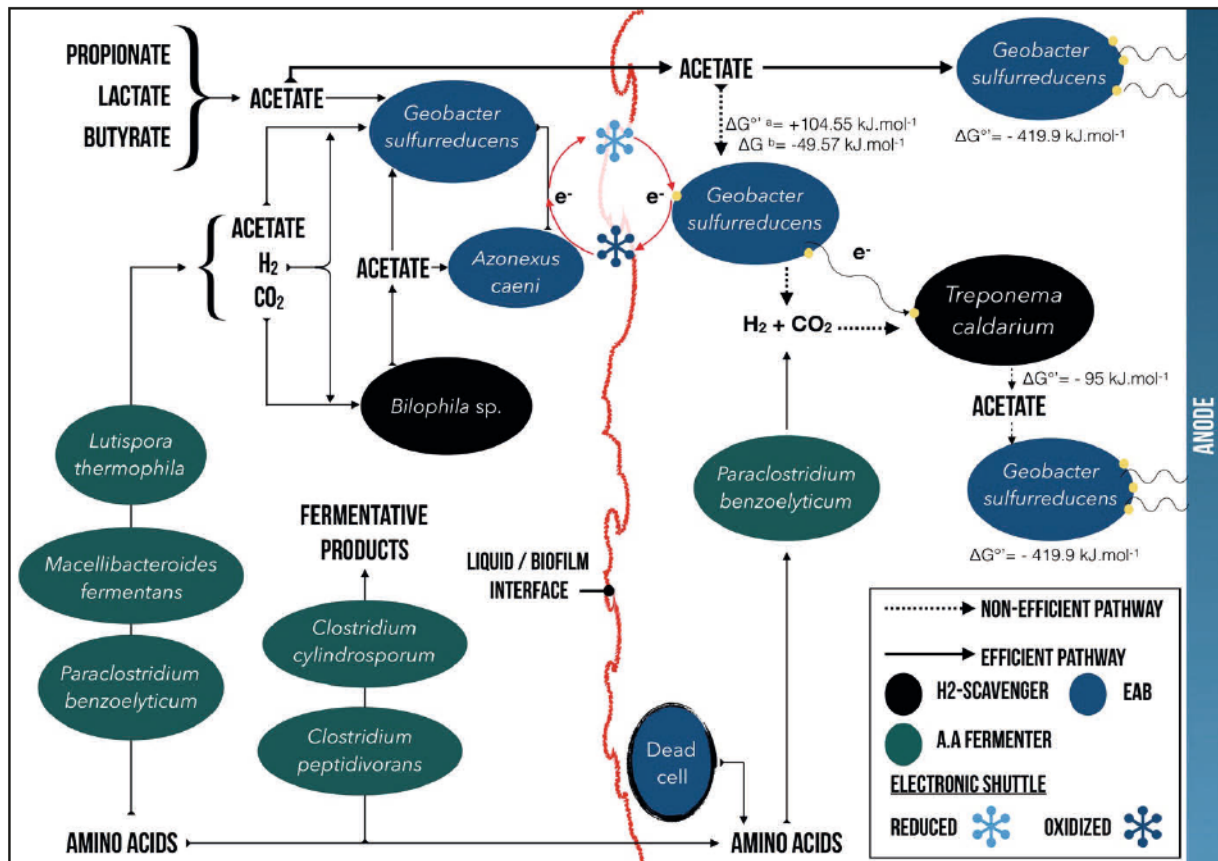
Performances indices	OTU 1	OTU 7	OTU 8	OTU 9
Current				
Density (A.m ⁻²)	-.32	-.74**	-.95***	-.79***
Coulombic efficiency (%)	.67**	.57*	.49	.19

3.2.5 Common bacteria in both core-microbiomes

Two bacteria were found common to both core-microbiomes i.e. bulk & biofilm: *P. benzoelyticum* & *G. sulfurreducens*. These bacteria were also significantly correlated in planktonic and anodic habitats with $r=0.82$ and $r=0.59$ respectively suggesting a high specificity of this bacterial couple on two different ecological habitats i.e. bulk and biofilm. As explained above, the oxidation of amino acids into organic acids, a known metabolism of *P. benzoelyticum*, seems favored by the presence of H₂-scavenger such as *G. sulfurreducens* which could establish syntrophies in biofilm and planktonic modes (Table 3-2). In biofilm lifestyle, *G. sulfurreducens* is able to use the anode as an electron terminal acceptor. In bulk, it can either use self-secreted electronic shuttles i.e. flavins, or use *P. benzoelyticum* redox active proteins i.e. c-type cytochromes as an electron terminal acceptor, which could explain the high specificity of this bacterial couple [56, 186]. Further analysis is needed to determine their precise ecological relationship.

3.2.6 Hypothetical core-microbiome metabolic network

Core-microbiome analysis (Biofilm & Bulk), potential metabolisms and correlations analysis (among OTUs and between OTUs and performance indices) allowed to establish a global metabolic network (Schematic 3-1). The network presents the eight OTUs of the planktonic core and the four of the anodic core. Acetate was the fermentation product that could be used directly at the anode by *G. sulfurreducens*. This metabolic pathway was the most direct because it involved only one OTU. Acetate in the medium could also be used by *G. sulfurreducens* or *A. caeni* using probably electronic shuttles as electron terminal acceptors. These shuttles could then be re-oxidized by the bacteria that were present in the biofilms. Concerning amino acids, they could ferment in acetate, H₂ and CO₂. Hydrogen and CO₂ could then be used by *Bilophia* sp. in an homoacetogenesis pathway. Within the biofilm, *T. caldarium* could be in contact with the *G. sulfurreducens* cells that were not connected to the anode in order to receive its electrons or the H₂ produced during the acetate oxidation. This pathway was the least efficient with respect to current density than the direct pathway because it involved homoacetogenesis.



Schematic 3-1 Hypothetical core-microbiome metabolic network. The yellow circles represent extracellular redox active proteins (RAPs). $\Delta G^{\circ' a}$ Gibbs free energy changes under standard conditions (25 °C, concentrations of reactants at 1 M or 1 atm) at pH 7.0. ΔG^b Calculations of free energy changes at 25 °C and pH 7.0 according to equation $\Delta G' = \Delta G^{\circ'} + RT \ln([\text{products}]/[\text{reactants}])$ and based on the following concentration: H₂, 10 ppmv in the gaseous state. $E^{\circ'}$ (RAP) = 254 mV vs SHE.

Conclusion

This study investigated the core-microbiomes of bacterial consortia growing on the biofilm and the planktonic part in the anodic chamber of microbial electrolysis cells. At the planktonic level, significant links between five of the eight OTUs were observed with particularly high

levels of correlations between amino acid fermenters and *G. sulfurreducens* ($r = 0.82-0.93$) suggesting trophic links. Interestingly, *Bilophila sp.* a well-known hydrogen-scavenger was connected to this OTU-network which probably indicating hydrogen production by this consortium. It would be interesting to understand the mode of electron transfer from the liquid medium to the electroactive biofilm. Concerning the anodic consortia, two hydrogen consumers were observed: *Treponema caldarium* and *Geobacter sulfurreducens* characterized as homoacetogen and electroactive respectively. A significant link has been shown between the abundances of *T. caldarium* and the current densities ($A.m^{-2}$), which seems consistent with the fact that the implementation of an homoacetogenic pathway is less favorable than direct oxidation of acetate to the anode by electroactive bacteria through the implementation of three metabolic steps against one. A correlation analysis between the OTUs showed a significant link between an hydrogen scavengers and an amino acid recyclers such as *Paraclostridium benzoelyticum*. This latter could benefit from a syntrophic association for the oxidation of amino acids. Further studies are needed to validate these hypotheses and better understand ecological relationships within bioelectrochemical consortia.

Ouverture

L'étude du core-microbiome associé aux écosystèmes électroactifs a permis d'isoler deux consortia différents : (1) le consortium électroactif associé aux biofilms, composé de quatre OTUs ; (2) le consortium planctonique composé de huit OTUs. Le regroupement de l'ensemble des échantillons a permis de réaliser des tests statistiques de corrélation grâce à l'amélioration de la précision de la p -value (passage de $n=4$ à $n=15$ échantillons). Ces tests de corrélation ont

été indispensables pour l'identification des OTUs d'intérêt. De manière intéressante, l'OTU 8 (identique à 99% à *Treponema caldarium*) était négativement corrélée ($r=-0.95$) aux densités de courant maximales, permettant l'utilisation de cette OTU en tant que bio-indicateur de CD_{max} . *T. caldarium* étant impliquée dans la voie biochimique d'homoacétogénèse, il semble probable que l'OTU 8 indique la mise en place de cette voie au sein de l'ensemble des biofilms électroactifs de cette étude. Une telle voie expliquerait la diminution du CD_{max} , car l'acétate ne serait plus uniquement oxydé par voie directe à l'anode par une bactérie électroactive, mais serait premièrement transformée en H_2 et CO_2 , étape limitante nécessitant la baisse de pression partielle de dihydrogène pour rendre favorable cette réaction. Des études complémentaires sont nécessaires pour vérifier cette hypothèse.

Concernant le consortium planctonique, l'étude a révélé des liens significatifs d'abondances sur 5 des 8 OTUs, groupées en trois catégories : (1) fermenteurs d'acides aminés ; (2) hydrogénotrophes ; (3) électroactifs.

Deux réseaux trophiques hypothétiques ont été proposés dans cette étude. Ces réseaux servent de base et doivent par la suite être vérifiés par d'autres méthodes analytiques comme des consortia synthétiques afin de valider/invalider et avancer vers une compréhension détaillée des écosystèmes électroactifs.

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Avant-propos

La problématique observée sur l'étude du chapitre 2 a mis en évidence une persistance du propionate au cours du temps lorsqu'il était en mélange avec de l'acétate (cas de la fermentation du lactate en acétate et propionate). L'analyse microbiologique a révélé une prédominance de *Geobacter toluenoxydans* en présence du propionate. Cette dernière était absente dans les MECs alimentées au lactate, où *Geobacter sulfurreducens* dominait. Comme *G. sulfurreducens* est incapable d'oxyder la propionate, l'hypothèse d'un 'effet barrière' a été envisagée. Une solution était nécessaire pour enrichir les biofilms anodiques avec des EABs spécialisées dans l'oxydation du propionate (comme *G. toluenoxydans*). Comme observé précédemment, le donneur d'électrons influence la structure des consortia bactériens anodiques. Par conséquent, des pré-acclimations à base de substrats ont été testées avec pour objectif de déterminer si des consortia anodiques pré-acclimatés étaient plus efficaces pour l'oxydation du propionate par rapport aux conditions non acclimatées. Dans ce cadre, une expérimentation a été mise en place, où des anodes ont été pré-acclimatées avec du propionate, pour les enrichir avec des EABs spécialisées dans l'oxydation du propionate avant de les transférer dans un milieu où seul du propionate était présent en tant que donneur d'électrons. Pour comparer les performances des MECs, des conditions de pré-acclimation anodiques à l'acétate et au lactate ont été

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implémentées. Des conditions de contrôle, c'est à dire sans pré-acclimatation, ont été effectuées pour vérifier si l'enrichissement était efficace pour lever l'effet barrière causé par *G. sulfurreducens* ou autre électroactive à faible versatilité métabolique et améliorer l'oxydation du propionate au cours du temps. Cette expérience est présentée en détail au chapitre 4.

ABSTRACT

Propionate has previously been reported as a recalcitrant substrate in microbial electrolysis cells (MECs). Only few electroactive bacteria (EABs) are able to use propionate as carbon source, such as *Geobacter toluenoxydans*, outcompeting *Geobacter sulfurreducens* for anode colonization. To avoid the dominance of *G. sulfurreducens*, unable to oxidize propionate, the emergence of specific propionate-oxidizing EABs could be promoted by applying appropriate pre-acclimation techniques. In this study, substrate-based pre-acclimations were implemented to (1) promote the emergence of specific propionate oxidizing EABs and (2) improve MEC performances (propionate oxidation kinetics, coulombic efficiencies and maximum current densities). The results show that anodic pre-acclimation with lactate or acetate was more effective than on propionate by slightly improving the coulombic efficiency ($\sim+9\%$). The community analysis revealed that the main bacteria was related to *Geobacter anodireducens*, capable of directly oxidizing propionate into current. However, this EAB was negatively correlated with the coulombic efficiency ($r=-0.74$). Propionate pre-acclimation promotes *G. anodireducens* which decreased CE, in contrast to acetate and lactate pre-acclimated conditions where some low-abundant bacteria showed good correlation with the CE. Among them, the presence of *Treponema caldarium* significantly correlated with the CE ($r=0.66$). This bacterium

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was previously described as homoacetogen and could have a role in the electroactivity of the anodic biofilm by oxidizing the hydrogen produced in propionate fermentation, and favoring thus an efficient conversion of propionate into current. Overall, these results indicate that subdominant bacteria together with dominant species could play an important role in electroactive biofilms.

Introduction

The consumption of fossil fuels has a direct impact on global climate change, making the use of renewable energies vital for the future. Among the environmentally friendly technologies able to produce renewable energy, bio-electrochemical systems (BESs) have gained interest over the past decade [2]. BES have been studied as alternatives to conventional wastewater treatment processes as they have the potential to reduce the energy demand with regards to traditional oxygenation in activated sludge processes [211]. Microbial electrolysis cells (MECs) use microorganisms as catalysts to oxidize organic substrates and provide electrons to produce hydrogen, using the anode as final electron acceptor. MECs represent a promising solution due to their ability to use various substrates present in wastewaters [153]. When acetate is used as a substrate, a voltage of 0.2 V is required for hydrogen production, which is significantly lower than the voltage of 1.8-2.0 V used for hydrogen evolution in water electrolyzers at ambient temperature [28]. Wastewaters contain organic matter but also soluble VFAs that accumulated by natural fermentation. Among these acids, propionate is used as a model substrate to study microbial syntrophies [207]. Moreover, as electron donor in MECs,

this substrate is poorly biodegradable, which is problematic from the perspective of a large-scale use of BESs [17, 139, 212].

Propionate and acetate accumulation in a 2:1 ratio is generally observed during lactate fermentation [7]. Acetate is rapidly oxidized (5 days) contrary to propionate that is persistent over time [17, 139, 207]. To better understand this persistence, microbiological analyses have previously revealed the predominance of *Geobacter sulfurreducens*, unable to oxidize this molecule, creating most probably a barrier effect at the anode (Flayac et al., 2018). This preponderance minimizes, if not excludes, the presence of other bacteria, resulting in a colonized anode able to convert acetate but not the other substrates [137]. When propionate is the sole electron donor, the presence of other EABs such as *Geobacter toluenoxydans* or *Geovibrio ferrireducens* was reported [13]. One solution to avoid the barrier effect of *Geobacter sulfurreducens* could be to pre-acclimate the anodic consortia using specific substrates. The purpose of such pre-acclimation step would be to establish a hierarchical ecosystem (fermentative and electroactive) with high a metabolic versatility for using many electron donors [93, 142, 145].

The aim of this study was to determine the impact of different anodic pre-acclimation strategies on the performances of MECs fed with propionate as final electron donor. In-depth analysis of the anodic microbial communities was performed to better understand the impact of the pre-acclimation steps on the microbial community structures and determine the link with MEC performances.

4.1 Materials & Methods

4.1.1 Inoculum

The microbial inoculum used in this work was sampled from the aeration tank of the Narbonne activated sludge wastewater treatment plant (11100, France). The latter was quickly used without storage at 1% v/v.

4.1.2 Operating of the MECs

All chemicals were of analytical or biochemical grade and purchased from Sigma-Aldrich. All potentials provided in this manuscript refer to the SCE reference electrode (KCL 3.0 M, +240 mV vs. SHE, Materials Mates, La Guilletière 38900 Sarcenas, France). All media prepared were adjusted to pH=7, flushed with high-purity N₂ gas (purity ≥ 99.995 %, Linde, France) for at least 30 min using air injection cannula. Bioelectrochemical experiments were conducted under potentiostatic control (BioLogic Science Instruments, France) with EC-Laboratory v.10.1 software and strictly anaerobic. The temperature was maintained at 37°C in a water bath for all incubations. A magnetic stirrer rotating a 350 rpm to homogenized the mixture. MEC tests were performed in quadruplicate with anodic potential fixed at +210 mV vs SCE.

4.1.3 Microbial electrolysis cell set up

The electrochemical system used for this experiment corresponded to a two-chambers cylindrical microbial electrolysis cell to avoid the presence of hydrogen from the cathode at the anodic compartment. Each chamber had a working volume of 900 mL. The anode was a 2.5 cm x 2.5 cm x 0.12 cm carbon plate (Mersen S.A, France), screwed onto a 2-mm diameter titanium rod (T1007910/13, Goodfellow SARL, France). The cathode was a grid 16 cm² 90% Platinum

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and 10% Iridium grid (Heraeus PSP., France). The MECs were hermetically sealed with a silicone and stainless steel ring at each chamber. Both chambers were separated with an anion exchange membrane (AEM, Fumasep FAA, FuMA-Tech BWT GmbH, Germany). Pre-acclimation was performed in batch with either propionate (PRO^{acc}), lactate (LAC^{acc}) or acetate (ACE^{acc}) as carbon source and electron donor. After the pre-acclimation phase (~10 and 20 days), the switch was set up. After the switch, propionate removal tests were performed over 7 days under three pre-acclimated conditions: PRO^{LAC} , PRO^{PRO} and PRO^{ACE} (Fig. 4-1). The controls correspond to a non-pre-acclimated anode operated for only one batch with either propionate ($\text{PRO}^{\text{control}}$), acetate ($\text{ACE}^{\text{control}}$) or lactate ($\text{LAC}^{\text{control}}$) as electron donor. When the current density ($\text{A} \cdot \text{m}^{-2}$) decreased to half its maximum value, the MEC was stopped and the electroactive biofilm was collected and placed under non-acclimated control conditions. For acclimation conditions, when the current density was close to half the maximum current density (during the first batch) only the anode was transferred to a new medium over a period of 7 days (batch 2). Before this transfer, a small piece (0.5 cm^2) of the electroactive biofilm was sampled from the anode.

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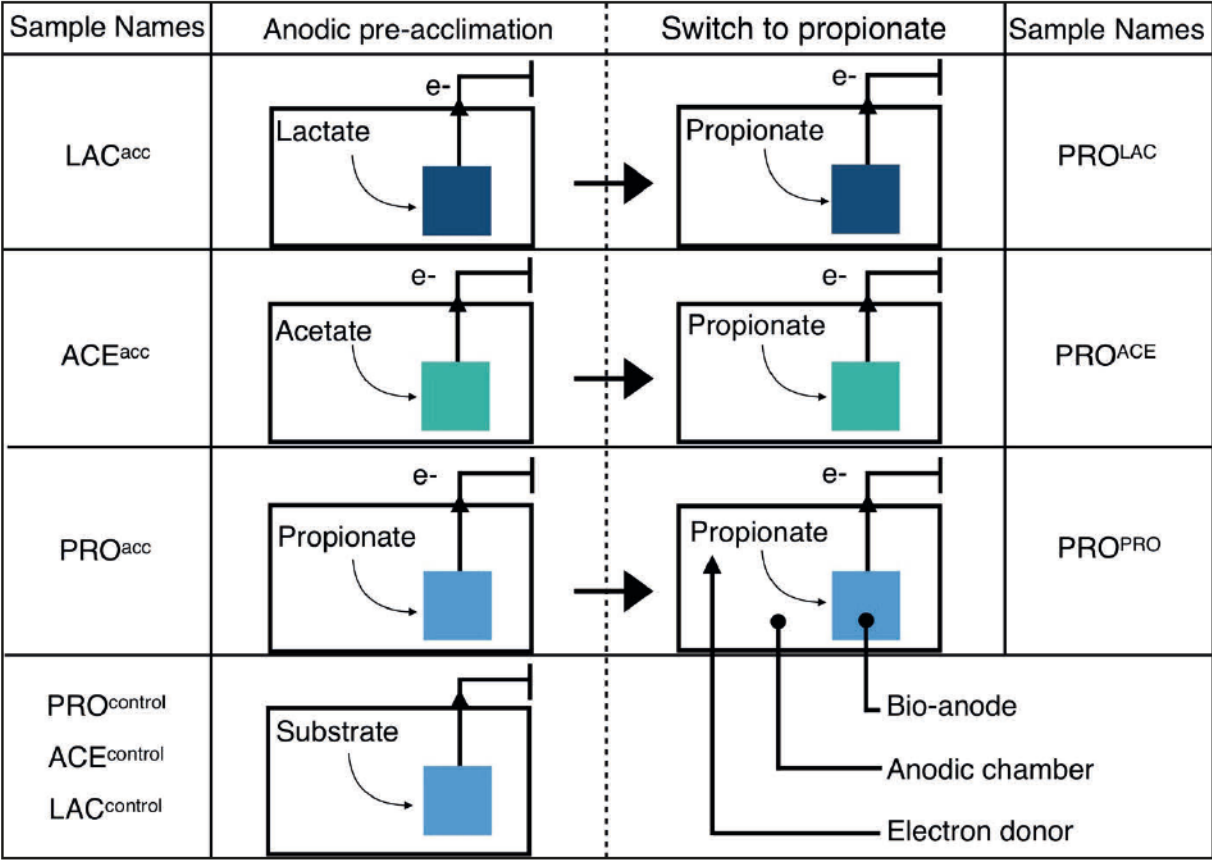


Fig. 4-1 Summary of the different pre-acclimations condition and the corresponding sample names. The test controls consist of a single batch. During the switch, only the anode was transferred to a new MEC with a new medium and the appropriate electron donor.

4.1.4 MEC Medium

The medium in the anodic chamber (per liter of water) was as follows: 0.5 g K₂HPO₄, 2.0 g NH₄Cl, 7.6 g MES buffer, 0.2 g yeast extract, 12.5 mL trace metal element solution 141 (DSMZ), and 2.11 g Sodium 2-bromoethanesulfonate (2-BES) was added to inhibit the methanogenic activity. The cathodic medium (per liter of water) contained 0.5 g K₂HPO₄, 7.6 g MES buffer and 12.5 mL trace metal element solution 141 (DSMZ). Acetate, lactate,

propionate and butyrate were separately used in anodic compartment as unique electron donor at a concentration of $\sim 80 \text{ m e}^- \text{ eq}$.

4.1.5 Analytical Methods

Concentrations of acetate, propionate, butyrate and lactate were determined by HPLC with a refractive index detector (Waters R410). Samples were first centrifuged at 13,500g for 15 min and the supernatants were then filtered at 0.2 μm . HPLC analysis was performed on an Aminex HPX-87, 300 x 7.8 mm (Bio-Rad) column operated at 35°C and at a flow rate of 0.4 mL/min with H_2SO_4 (4 mM) as mobile phase. For each batch, the planktonic part was sampled after inoculation as the starting point, constituting the inoculum samples. At the end of each batch, the planktonic part was collected and constituted the bulk samples, while the anodic biofilm was harvested with a blade. These three types of samples (Inocula, bulks and biofilms) were centrifuged at 13,500g for 15 min and the pellet was stored at -20°C prior to microbial community analyses.

4.1.6 Microbial Community Analysis

DNA extraction was carried out with QIAamp fast DNA stool mini kit in accordance with the manufacturer's instruction (Qiagen, Hilden, Germany). DNA extraction was confirmed using Infinited 200 PRO Nanoquant (Tecan Group Ltd., Männedorf, Switzerland). Amplicons from the V3 to V4 regions of 16S rRNA genes were amplified with bacterial forward 343F 5'-TACGGRAGGCAGCAG-3'; (Liu et al., 2007) and reverse 784R 5'-

TACCAGGGTATCTAATCC-3'; (Anderson et al., 2008) primers. Adapters were added for multiplexing samples during the second amplification step of the sequencing. The PCR mixtures (50 µl) contained 0.5 U of Pfu Turbo DNA polymerase (Stratagene) with its corresponding buffer, 0.5 mM of each primer, 200 mM of each dNTP and 10 ng of genomic DNA. Reaction were carried out in a Mastercycler thermal cycler (Eppendorf) as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min. The size and amount of PCR products were defined using a Bioanalyser 2100 (Agilent). The community composition was evaluated using the MiSeq v3 chemistry (Illumina) with 2 x 300 bp paired-end reads at the Genotoul platform (www.genotoul.fr). Sequence were retrieved after demultiplexing, cleaning, and affiliating using Mothur [165]. All sequences were submitted to Genbank under accession numbers MH465676-MH468681.

4.1.7 Quantitative PCR (qPCR)

PCRs were prepared using 96-well real time PCR plates (Eppendorf, Hamburg, Germany) and Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). After, 6.5 µl of Express qPCR supermix with premixed ROX (Invitrogen, France), 2 µl of DNA extract with three appropriate dilutions, 100 nM forward primer F338-354 (5'-ACTCCTACGG GAGGC AG-3'), 250 nM reverse primers R805-785 (5'-GACTA CCAGG GTATC TAATC C-3'), 50 nM TaqMan probe and water were added to obtain a final volume of 12.5 µl for all analyses. A first incubation of 2 min at 95°C followed by 40 cycles of denaturation (95°C, 7 s; 60°C, 25 s) was performed. From each assay, one standard curve was generated by using tenfold dilution in sterilized water

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(Aguettant Laboratory, Lyon, France) of a target plasmid (Eurofins Genomics, Germany). The initial DNA concentrations were quantified using the Infinite 200 PRO NanoQuant (Tecan, France).

4.1.8 Calculations

Coulombic efficiency (CE) was calculated as the percentage of electrons that passed through the circuit in a single batch test against the amount of electrons that were initially available in the substrate [1].

4.1.9 Statistical Analysis

All statistical analyses were carried out in R version 3.2.3 (R core Team 2014). The differences between current densities and coulombic efficiencies between the conditions were tested with one-way ANOVA followed by Tuckey's HSD post-hoc test with a statistical significance (P -value) < 0.05 . Weighted-UniFrac distance-based PCoA ordination was used to represent inter-samples distances with phyloseq package [166]. The Monte Carlo simulation was used to test the significant difference between microbial composition according to the conditions with P -value of 0.001.

4.2 Results and discussion

4.2.1 MECs performances (CD_{max} , CE & P_{drop}^{7d}).

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To determine whether an anodic substrate-based pre-acclimation could improve the decrease in propionate for seven days (or P_{drop}^{7d}), CD_{max} and CE, pre-acclimation tests were carried out. The mean CD_{max} values were $1.25 \pm 0.5 \text{ A.m}^{-2}$, $1.52 \pm 0.47 \text{ A.m}^{-2}$, $4.13 \pm 2.28 \text{ A.m}^{-2}$, $3.36 \pm 2.16 \text{ A.m}^{-2}$ and $1.94 \pm 1.03 \text{ A.m}^{-2}$ for the conditions $PRO^{control}$, PRO^{acc} , PRO^{PRO} , PRO^{LAC} and PRO^{ACE} respectively. As a high variance was observed in CD_{max} , with a difference of 72.7% between the lowest and highest value for the same PRO^{PRO} conditions (Fig. 4-3), no significance difference was observed whatever the condition (Table 1). CD_{max} measures the maximum rate of electrons uptake from propionate oxidation by the electroactive biofilm. Thus, anodic pre-acclimations have no significant impact on this performance index.

Propionate degradation tests were performed over a seven-day period (P_{drop}^{7d}), with the three acclimated conditions. Propionate concentrations over time can be observed (Fig. 4-2). The mean values of P_{drop}^{7d} were $0.19 \pm 0.02 \text{ g.L}^{-1}$, $0.15 \pm 0.07 \text{ g.L}^{-1}$, $0.12 \pm 0.06 \text{ g.L}^{-1}$ for the conditions PRO^{PRO} , PRO^{LAC} and PRO^{ACE} respectively. No difference in P_{drop}^{7d} was observed whatever the condition which is consistent with CD_{max} results, i.e. no significant difference (Table 4-1). Therefore, pre-acclimations of anodic consortia were ineffective with respect to the propionate oxidation rate as a single electron donor (Table 4-1).

The mean values of coulombic efficiency were $73.6 \pm 1.26\%$, $75.3 \pm 1.38\%$, $84.0 \pm 0.88\%$ and $83.1 \pm 1.13\%$ for the conditions $PRO^{control}$, PRO^{PRO} , PRO^{LAC} and PRO^{ACE} respectively. Coulombic efficiencies were significantly higher for PRO^{LAC} and PRO^{ACE} conditions with regards to PRO^{PRO} and $PRO^{control}$ (Table 4-1). Electrons can be stored in biomass or other uncharacterized sinks, resulting in a decrease of the CE [65]. Such significant differences may

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be due to less electrons stored in the biomass for acetate- and lactate-pre-acclimated bio-anodes when compared to the other two conditions. Therefore, lactate and acetate pre-acclimation were more effective than propionate pre-acclimation and improved the recovery of electrons from propionate degradation after the switch.

Table 4-1 Summary of the different performance indices (CD_{max} , CE and P_{drop}^{7d}) of MECs fed with propionate. P_{drop}^{7d} = Propionate drop during seven days. The exponent letters indicate the levels of significance. ‘ns’ means not significant.

MECs	Condition	First substrate	Second substrate	CD_{max} (A.m ⁻²)	CE (%)	P_{drop}^{7d} (g.L ⁻¹)
PRO ^{control}	No-acclimated	Propionate	-	1.25±0.5 ^(ns)	73.6±1.26 ^(a)	-
PRO ^{PRO}	Switchless	Propionate	Propionate	4.16±2.28 ^(ns)	75.3±1.38 ^(a)	0.19±0.02 ^(ns)
PRO ^{LAC}	Acclimated	Lactate	Propionate	3.36±2.16 ^(ns)	84.0±0.88 ^(b)	0.15±0.07 ^(ns)
PRO ^{ACE}	Acclimated	Acetate	Propionate	1.94±1.03 ^(ns)	83.1±1.13 ^(b)	0.12±0.06 ^(ns)

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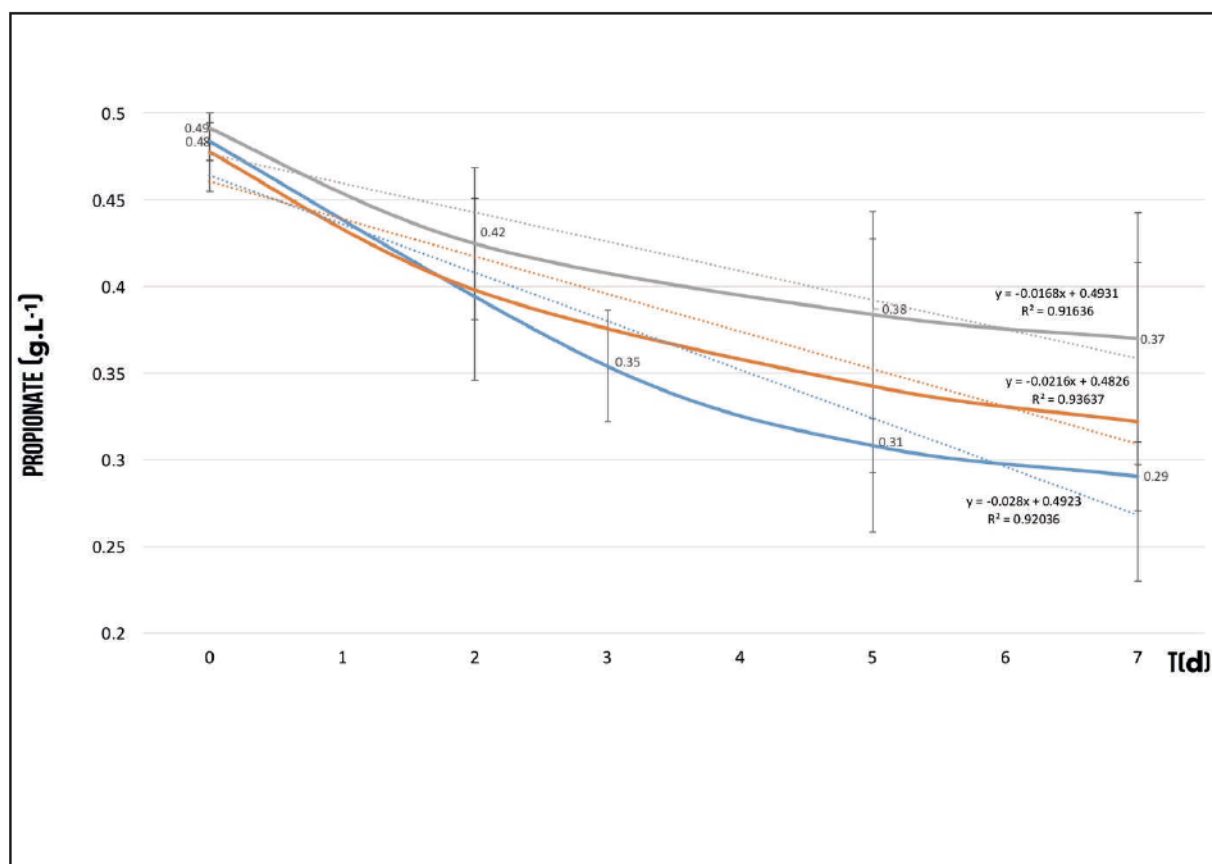


Fig. 4-2 Propionate concentration over time of conditions PRO^{PRO} in blue (■), PRO^{LAC} in orange (■) and PRO^{ACE} in grey (■).

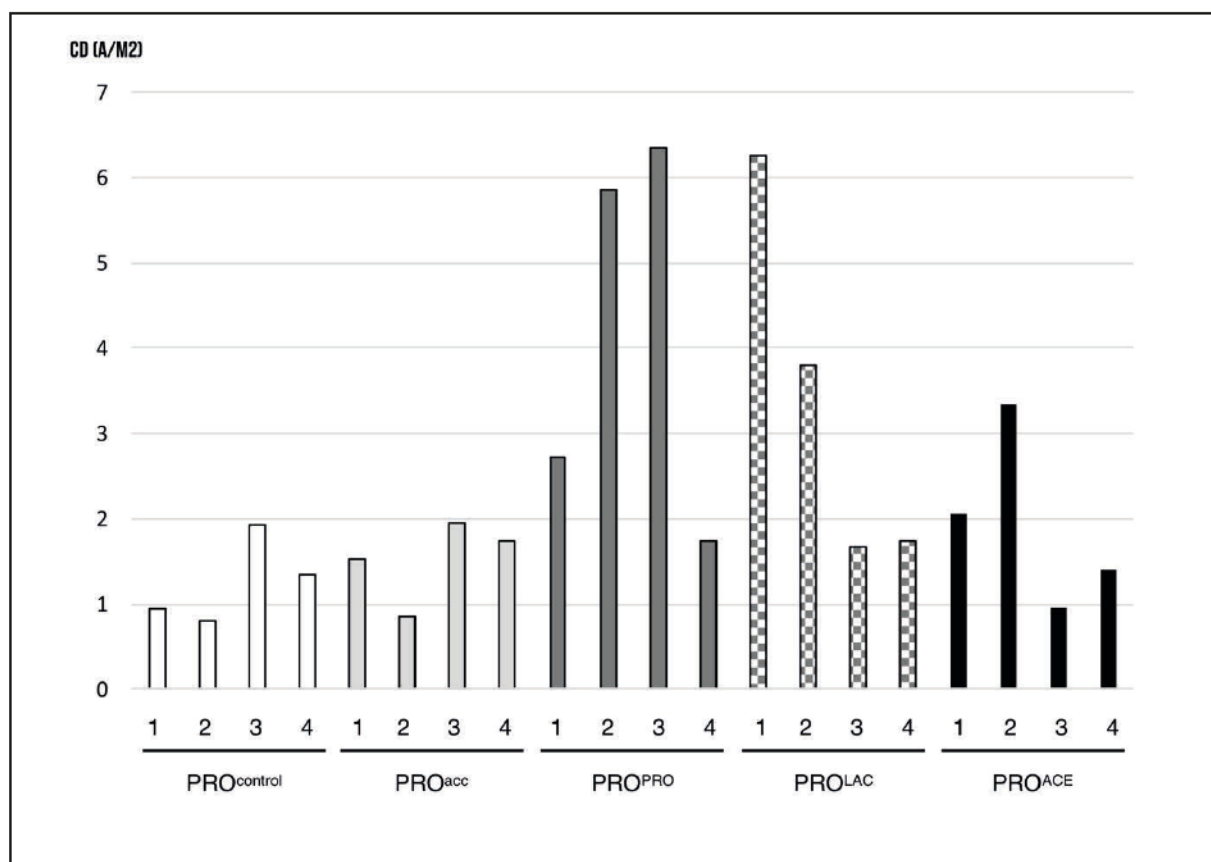


Fig. 4-3 Maximum current densities per sample. The associated numbers indicate the replicate number.

4.2.2 Microbial communities analysis

High-throughput sequencing of 16S rRNA gene was used to characterize the bacterial communities of the anodic biofilms and the bulk sampled from the sixteen MECs at the end of the experiments. 16S rRNA gene library had 3,257,802 high-quality reads (average length ~406 bp) after treatment (denoising, quality filtering and removal of chimeric sequences). The sequences were assigned to OTUs with a $\geq 95\%$ sequence identity threshold.

4.2.2.1 Microbial diversity analysis

Principal Coordinate Analysis (PCoA) based on weighted-UniFrac distance matrix was used to determine whether the microbial structures were different according to the pre-acclimation of anodic consortia. (Fig. 4-4). On this ordination, 80.2% of the total variance is represented on both axes. Concerning samples distances, PCoA analysis did not indicate significant difference between pre-acclimated (PRO^{acc} , LAC^{acc} , ACE^{acc}) and acclimated (PRO^{PRO} , PRO^{LAC} , PRO^{ACE}) conditions contrary to other studies using simple substrate pre-acclimation [142, 154, 156]. The control conditions ($PRO^{control}$, $ACE^{control}$ and $LAC^{control}$) were not significantly different from the acclimated conditions indicating a low variability of the main community structures. Concerning consortia, two replicates of the conditions PRO^{ACE} and PRO^{LAC} , present to the right of the axis 1 are close to the positions of the *Lentimicrobium* sp. and *Treponema* sp. However, the majority of the samples are close to the center of the two axes, near to the position of *Geobacter anodireducens*, *Lustispora thermophila* and *Clostridium acidisoli*. Thus, the PCoA analysis does not show a specific taxon to a given condition which indicates a non-specific selection of consortia through pre-acclimation.

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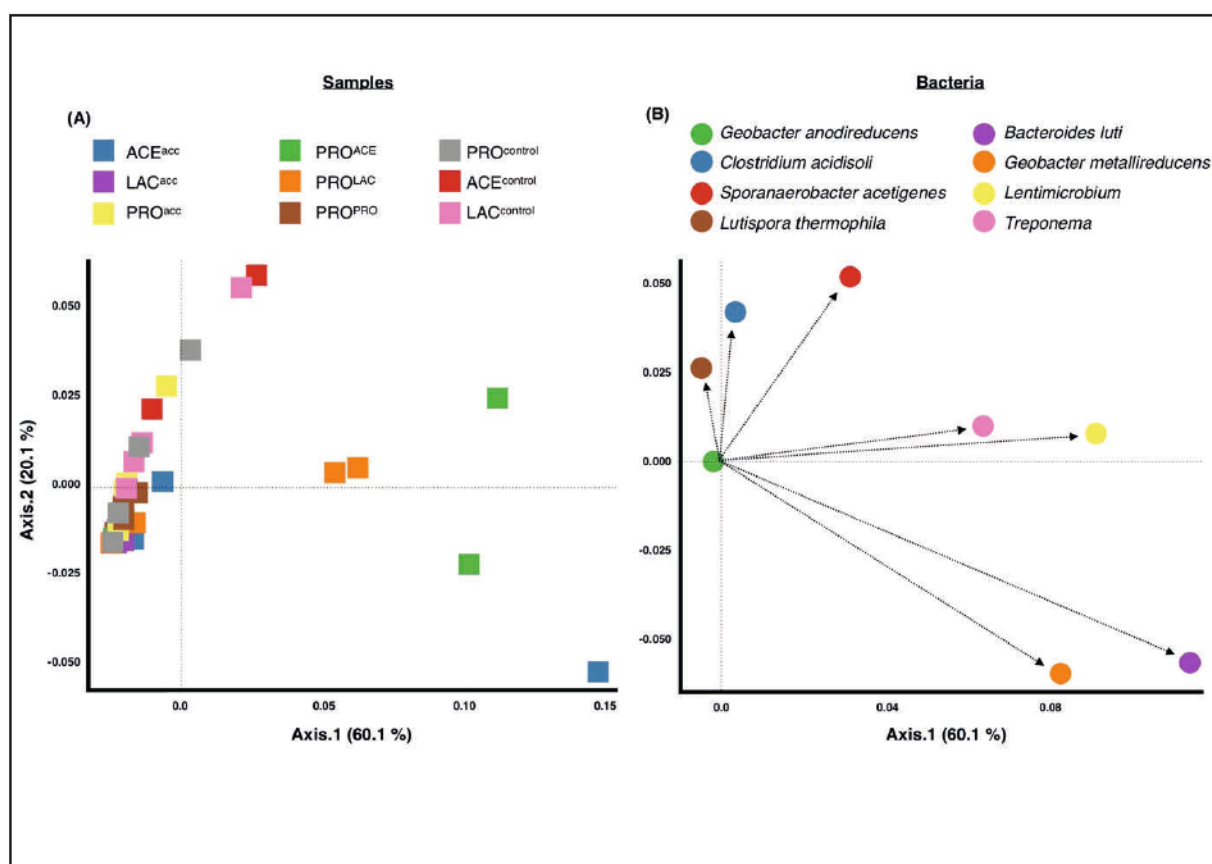


Fig. 4-4 Principal coordinate analysis (PCoA) based on weighted-UniFrac distance matrix showing: (A) The distribution pattern of the samples. (B) Taxa scores of the majority bacteria that explain some of the variance between principle coordinates axes (scale of eigenvector is relative to the influence of that taxa to overall discrimination).

To determine the differences in diversity between the anodic consortia of the different conditions, a Shannon index was calculated (Fig. 4-5). The Shannon index gives access to the specific diversity of each sample according to the number of species (species richness) and their distribution (specific equitability). The Shannon index shows that the PRO^{LAC} and PRO^{ACE} conditions had a significantly higher diversity compared to LAC^{acc} with a diversity of 0.91 ± 0.10 , 1.24 ± 0.56 and 0.47 ± 0.17 respectively. PRO^{LAC} had also a higher diversity compared

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to $\text{LAC}^{\text{control}}$ (0.65 ± 0.12) (Fig. 4-5). The other conditions were not significantly different from each other with an average diversity of 0.64 to 0.82.

Shannon indices reveal an increase in diversity when the electron donor changed from lactate (LAC^{acc}) to propionate (PRO^{LAC}). However, whatever the conditions, the Shannon index remains low compared to similar ecosystems, with means below 1.24, indicating little overall diversity and a dominance of one or few species compared to others electroactive biofilms [213].

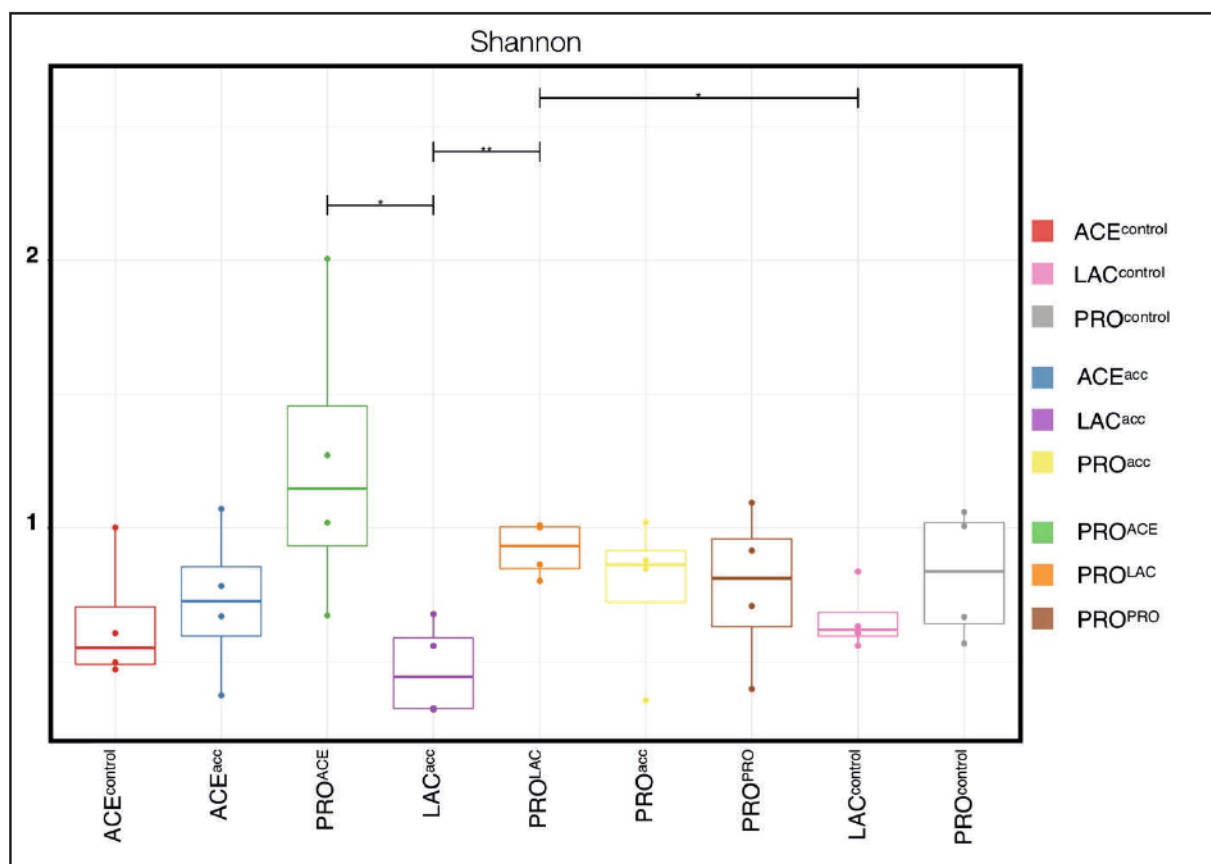


Fig. 4-5 Shannon diversity of the anodic biofilms according to the conditions.

4.2.2.2 Anodic bacterial communities of the acclimated bio-anodes.

The distribution of species is presented Fig. 4-6. OTUs were selected according to an abundance filter, i.e. at least a relative abundance of 5% in one sample. All samples, whatever the conditions, were dominated by *G. anodireducens*. The increase in the Shannon index (0.47 ± 0.17 to 0.91 ± 0.10) observed between LAC^{acc} and PRO^{LAC} can be explained by a slight

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decrease of *G. anodireducens* as well as an increase of *Treponema* sp., *Lentimicrobium* sp. and the minority OTUs (Others).

G. anodireducens (OTU 1) is a well-known electroactive bacterium capable of using lactate, acetate, propionate and hydrogen as electron donors (Table 4-2). Its predominance in all samples was probably due to its high metabolic versatility. This OTU seems to be a key player in all communities. Indeed, in addition to being able to use the organic molecules tested (before & after the switch), *G. anodireducens* is able of using the anode as sole electron terminal acceptor [214]. Thus, the high metabolic versatility of this bacterium could explain why no significant difference of communities between pre-acclimated (i.e. before switch) and acclimated anodes was observed (Fig. 4-4). It can be concluded that, whatever the conditions of pre-acclimation (acetate, lactate or propionate pre-acclimation), the communities were not impacted because all of them were dominated by OTU 1 (*G. anodireducens*). So, a similar structure of the communities could explain that, in our case, substrate pre-acclimation had no effect on the P_{drop}^{7d} (Table 4-1). Concerning CD_{max} , the high variances observed (Fig. 4-3) are misunderstood since all anodes present a very similar microbial structure. Only the CE was significantly different between the conditions (Table 4-1).

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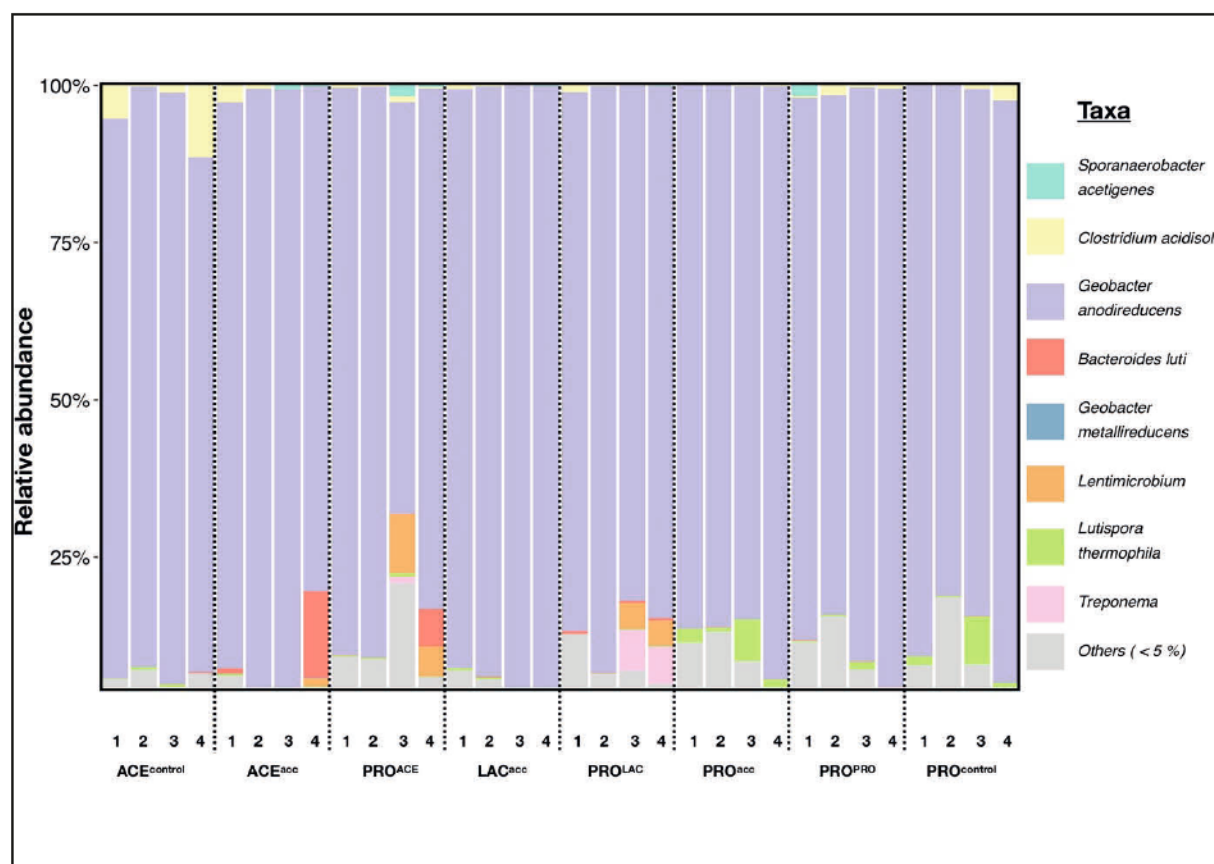


Fig. 4-6 Relative taxa abundances at Species level in anodic biofilms. Numbers (1-4) are specific to the replicate.

Table 4-2 Major bacteria (more than 5% in at least one sample) with their probable metabolic reactions.

Taxa	OTU [% id]	Electron donors	Metabolic products	Ref.
<i>Sporanaerobacter acetigenes</i>	32 [100]	Peptides and amino acids	Acetate, H ₂ , CO ₂	[215]
<i>Clostridium acidisoli</i>	21 [95]	?	?	[204]

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<i>Geobacter</i> <i>anodireducens</i>	1 [99]	Lactate, Propionate, Acetate, H ₂	e ⁻ , H ⁺ , CO ₂	[214]
<i>Bacteroides luti</i>	4 [98]	?	?	[216]
<i>Geobacter</i> <i>metallireducens</i>	39 [99]	Lactate, Propionate, Acetate, H ₂	e ⁻ , H ⁺ , CO ₂	[97]
<i>Lentimicrobium</i>	20 [95]	?	?	[217]
<i>Lutispora</i> <i>thermophila</i>	9 [99]	Peptides and amino acids	Acetate, Propionate, Iso-Butyrate, Iso- Valerate	[189]
<i>Treponema</i>	54 [95]	H ₂ , CO ₂	Acetate	[176]

Coulombic efficiency was the only significantly different parameter between the tested conditions i.e. ~9% higher for PRO^{LAC} and PRO^{ACE} compared to PRO^{PRO}. To determine which OTU(s) can improve the CE, a correlation analysis was performed. This correlation takes into account the absolute abundance of OTUs and the CE values (Table 4-3). Bacteria were previously selected according to an abundance filter of 0.5% in at least one sample in order to take into account minority bacteria. A total of 41 bacteria were selected with this abundance filter. Among them, only five were significantly correlated with CE.

G. anodireducens, the main EAB was negatively correlated with the CE ($r=-0.74$). This relationship suggests an electron leakage during the conversion of propionate into current. To be converted into electricity, propionate is first fermented into acetate and hydrogen [17, 207]. Such an electron leakage may occur during this reaction due to a loss of hydrogen either by

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oxidation and storage of electrons in biomass, or by volatilization [218]. Inversely, *Treponema caldarium* was positively correlated with the CE ($r=0.66$). This species supports homoacetogenesis by producing acetate from H_2 and CO_2 [176, 201]. As previously demonstrated, hydrogenotrophs are linked to electroactive bacteria when they are in biofilms by making thermodynamically favorable hydrogen-producing fermentations [66]. On propionate-fed bio-anodes, it seems that *T. caldarium* played a key role, probably by promoting the oxidation of propionate to H_2 , CO_2 and acetate by recycling H_2 and producing acetate via an homoacetogenic pathway. These reactions would redirect the electrons from H_2 into current and thus increase the CE (Schematic 4-1). Among the five OTUs identified, three were close to bacteria known to ferment amino acids into organic acids (OTUs 3, 6 & 59). *L. thermophila*, *M. fermentans*, *A. hydrogenigenes* were negatively correlated with CE with $r=-0.72$, $r=-0.63$ and $r=-0.74$ respectively. The fermentation of amino acids to organic acid produces hydrogen during fermentation with an hydrogen scavenger bacterium [205, 206]. The latter is therefore immediately used, probably in the form of biomass leading to losses of electrons a decrease in CE.

To go further, the other two indices, CD_{max} and P_{drop}^{7d} were correlated with bacterial abundances of more than 0.5% in at least one sample. Indeed, even if these indices were not significantly different between conditions (Table 4-1), there may be interdependent relationships with individual points. Therefore, these correlations can reveal interdependencies regardless of the condition tested (Table 4-3). A total of 41 bacteria were selected with this abundance filter. Among them, only five were significantly correlated with CD_{max} or P_{drop}^{7d} .

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M. fermentans and *A. hydrogenigenes* were positively correlated with the CD_{max} with $r=0.71$ and $r=0.59$ respectively. Both were also negatively correlated to the CE. Thus, the fermentation of amino acids, from yeast extract and probably dead cells, could increase the CD_{max} probably by producing hydrogen or acetate, the main fermentative products directly usable by *G. anodireducens* [206]. However, as described above, this fermentative pathway involves a syntrophic microorganism that uses hydrogen to form biomass and could lower CE [167, 188, 219].

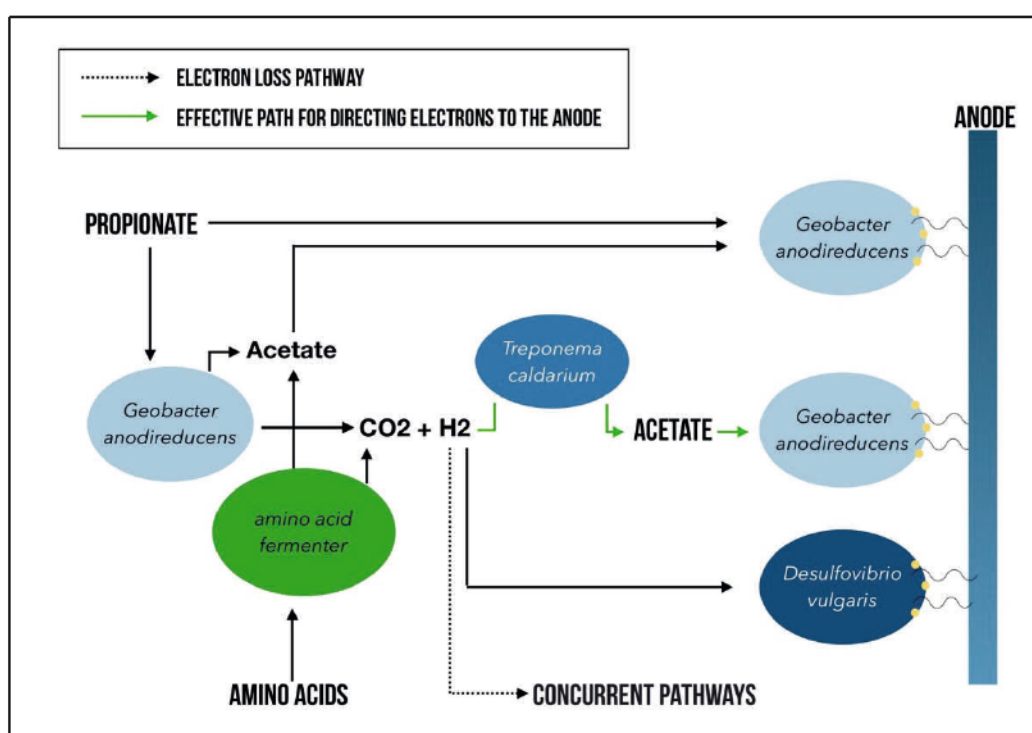
Desulfovibrio vulgaris was positively correlated to CD_{max} ($r=0.64$). This bacterium is capable of oxidizing insoluble minerals such as Cr(VI) or U(VI) with H_2 as electron donor using c3-cytochromes for exoelectrogenesis [105]. Within the same genus, *D. desulfuricans* was characterized electroactive in a mediator less microbial fuel cell [220]. *D. vulgaris* could therefore be electroactive and thus capable of improve CD_{max} when propionate was present as an electron donor by probably by oxidizing hydrogen. The summary of the putative network is available in Schematic 4-1.

Table 4-3 Correlations (*Pearson* index) between bacteria that were more than 0.5% present in at least one sample and performance indices. * p value < .05, ** p value < .01. P_{drop}^{7d} =Propionate drop during seven days.

OTU [%id]	Taxa	CD_{max}	CE	P_{drop}^{7d}
1 [99]	<i>Geobacter anodireducens</i>	.48	-.74**	.09
3 [96]	<i>Lutispora thermophila</i>	.44	-.72**	.13
6 [100]	<i>Macellibacteroides fermentans</i>	.71**	-.63*	.35

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13 [97]	<i>Petrimonas sulfuriphila</i>	-.45	.35	-.72**
19 [99]	<i>Desulfovibrio vulgaris</i>	.64*	-.48	.24
26 [99]	<i>Treponema caldarium</i>	-.46	.66*	-.56
59 [97]	<i>Acetobacteroides hydrogenigenes</i>	.59*	-.74**	.24
61 [98]	<i>Proteocatella sphenisci</i>	.68*	.07	.41



Schematic 4-1 Hypothetical propionate degradation network.

Conclusion

In this study, substrate based pre-acclimations were performed to determine their impact on CD_{max} , CE and propionate degradation kinetics. Acetate, lactate and propionate were

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separately used to pre-acclimate bio-anodes that were then tested with propionate over a seven-day period. No significant improvement was observed for the CD_{max} and P_{drop}^{7d} indices. Nonetheless, CE was significantly improved after acetate and lactate pre-acclimation with regards to propionate pre-acclimated bioanodes. Community analysis revealed that the most dominant species was *Geobacter anodireducens*, capable of direct oxidization of propionate into current. However, this species was negatively correlated with the coulombic efficiency ($r=-0.74$). Propionate pre-acclimation promoted this bacterium which therefore decreased the CE when compared to acetate and lactate pre-acclimated conditions. The latter conditions favored the presence of minority bacteria. Among them, *Treponema caldarium* was significantly correlated to CE ($r=0.66$). This species was previously described as homoacetogen and could have an important role in electroactive biofilm by oxidizing the hydrogen produced during propionate fermentation. All together, these results indicate that minority bacteria could have an important role in electroactive biofilms.

Ouverture

L'expérience de pré-acclimation a montré aucune amélioration significative de CD_{max} et de la vitesse d'oxydation du propionate sur une période de 7 jours (P_{drop}^{7d}). Seul le rendement Faradique a été amélioré concernant les consortia anodiques pré-acclimatés au lactate et à l'acétate comparé à l'acclimation au propionate avec une amélioration de ~9%. L'analyse des communautés bactériennes a révélé la présence en majorité d'une bactérie, *Geobacter anodireducens*, capable d'oxyder le propionate, l'acétate, le lactate et l'hydrogène en utilisant l'anode en accepteur terminal d'électrons. Pour comprendre les raisons de

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L'amélioration des rendements Faradiques, des analyses de corrélations ont révélées que *G. anodireducens* était négativement corrélée à cet indice ($r=-0.74$) alors que *Treponema caldarium* y était positivement corrélée ($r=0.66$). *T. caldarium* est caractérisée en tant qu'homoacétogène et permettrait d'après son métabolisme une meilleure utilisation de l'hydrogène, produit pendant la fermentation du propionate en formant de l'acétate qui est ensuite préférentiellement utilisé par *G. anodireducens*. Par conséquent, cette étude montre que même en présence d'une EAB à haute versatilité métabolique, cette dernière pourrait être inefficace vis à vis du rendement Faradique. La présence d'une bactérie homoacétogène sous-dominante au sein des consortia électroactifs semble efficace vis à vis du rendement Faradique. Une coculture permettrait de valider cette hypothèse afin de mieux comprendre les liens écologiques précis entre ces deux bactéries.

5 Chapter 5. On the choice of a proper inoculum for treating industrial wastewater with BES

Avant-propos

Les études d'écologie microbienne des chapitres 2 à 4 ont été réalisées avec des substrats définis dans l'objectif de réduire la complexité des écosystèmes notamment en termes de relations interspécifiques afin de mieux comprendre les mécanismes écologiques fondamentaux. Par exemple, il a été précédemment mis en évidence des liens étroits entre les espèces électroactives du genre *Geobacter* et une homoacétogène, *Treponema caldarium*. Cependant avec l'utilisation d'eaux usées réelles en tant que substrat, quelle est la différence de diversités des consortia par rapport à ceux alimentés avec des substrats définis ? En effet, pour l'utilisation des BESs dans le cadre du traitement des eaux usées, ces dernières seront utilisées en conditions réelles. Il est donc important de déterminer les similarités et les différences entre les études effectuées en conditions synthétiques et celles en milieux complexes. Pour aborder cette question, des expérimentations ont été mises en place avec une eau usée issue d'une usine fabriquant une boisson à base de lait. L'objectif était de mettre en place des consortia différents, et de développer le consortium microbien le plus efficace pour traiter les eaux usées industrielles avec trois inocula différents. Le premier était composé de boues d'une eau usée industrielle, le second d'eaux usées urbaines et le troisième d'une culture enrichie en bactéries électroactives (à partir de la partie liquide d'une MFC alimentée avec de l'acétate sur du long-terme). Ces inocula ont été utilisés soit seul, soit dans différents mélanges de deux ou trois. Une condition

contrôle, sans ajout d'inoculum a été mise en place. Selon les différents inocula utilisés, les MFCs ont montré des comportements différents en termes d'indices de performance comme le rendement Faradique, la densité maximale de courant (CD_{max}) et la DCO éliminée. Les réacteurs inoculés avec les boues industrielles ont surpassé tous les inocula et la combinaison d'inocula testée, produisant une CD_{max} de $0,65 \pm 0,04 \text{ A/m}^2$, avec une CE de $70 \pm 18\%$ et $77 \pm 10\%$ de DCO éliminée. L'étude des consortia électroactifs a mis en évidence la contribution positive du genre *Geobacter* sp. vis à vis des densités de courant.

ABSTRACT

Bioelectrochemical systems (BES) are a promising emerging technology for resource recovery from organic waste. However, as only few simple substrates can be utilized by electroactive bacteria (EAB), an efficient bioconversion of complex organic substrates (such as those contained in industrial wastewater) requires the establishment of an effective syntrophic consortium between fermenters and EAB. With the objective of developing the most effective microbial consortium to treat industrial wastewater, the use of sludge from industrial wastewater treatment plant as inoculum was compared with anaerobic sludge from urban wastewater treatment plant, as the most common used inoculum in BES, and with an EAB enriched culture. Different mixtures, of the three inocula and the use of the wastewater itself were evaluated. The acclimation procedure was carried out in MFC mode. Fourteen identical air-cathode MFCs were fed with a wastewater sampled from a facility producing a milk-based drink. According to the different inocula used, MFCs showed different behaviors according to the performance indices considered, such as coulombic efficiency (CE), maximal current

density (CD_{max}) and COD removal. The reactors inoculated with the sludge from the industrial sludge out-performed all the tested inocula and combination of inocula, producing a CD_{max} of $0.65 \pm 0.04 \text{ A/m}^2$, with a CE of $70 \pm 18\%$, and $77 \pm 10\%$ of COD removed.

An in-depth microbial ecology analysis was carried-out with the objective of investigating the relationships existing between the diversity of mixed-species microbial consortia, their electrogenic potential and their effectiveness in treating industrial wastewaters in BES. The MFC inoculated with the sludge from the industrial sludge contained more *Geobacter* sp. in the anodic biofilms than other conditions. *Geobacter* sp. was positively correlated with CD_{max} ($r = 0.67$) and CE ($r = 0.73$) indicating its effectiveness in improving the performances of the MFCs fed with industrial wastewaters. New insights were provided giving some guidelines to enhance the selection of effective electricity-driving communities for industrial wastewaters treatment. The results allow a better understanding of the impact of the inoculation strategy on MFC performances and associated communities.

Introduction

Clean energy production is the global development goal of this century. In this context, fossil energy must be replaced by renewable energy in order to limit global change and preserve the stability of the ecosystems, their diversity and the essential biogeochemical cycles. Microbial fuel cells (MFCs) are one type of bioelectrochemical systems (BESs) capable of degrading the soluble organic matter and produce electric power, using bacteria as biocatalyst [161, 221-223]. BES are promising system for renewable energy production since they can use wastewaters as

substrates. Complex organic matter, such as the one contained in waste streams, is first fermented into simple substrates such as short chain fatty acids which are then used by bacteria called electroactive, i.e. capable of transferring electrons to the anode via extracellular electron transfer (EET) [224]. In MFC, the electrons, once recovered at the anode, pass through the circuit composed of a resistance to the cathode where they react with oxygen and protons. The reduction of oxygen in water is a thermodynamically favorable reaction allowing energy production in electric form [2].

The use of microbial fuel cells for the treatment of real effluents requires the consideration of many parameters to achieve optimal performances [223]. These parameters are classified into three categories with (1) the design of the system, (2) the operating parameters and (3) the biological parameters. The system design may include the type of membrane (e.g. anionic or cationic membrane) or a membrane-less reactor, the anode material (e.g. carbon, steel, graphite) as well as its shape (e.g. plate, brush, powder, mesh, cloth) or the type of reactor architecture (e.g. cubic or tubular) [225-229]. The operating parameters to be considered can be external resistance, pH, temperature, conductivity as well as feeding mode (e.g. batch, feed-batch or continuous) [230, 231]. The biological parameters can include: the type of the culture/inoculum as pure culture or mixed consortium, the addition of electronic shuttles (e.g. flavins) or quorum sensing auto-inducers, the anodic pre-acclimations (e.g. by the use of specific substrates) or in the case of mixed culture the source of inoculum (e.g. anaerobic sludge, sediment) [232-234]. When working with mixed cultures, biological parameters still represent the less understood variable because of the presence of many interconnected species allowing the production of a flux of electrons from the degradation of complex substrates [211, 235, 236]. Microbial ecology

studies have shown that EABs are ubiquitous and in interaction with different species according to ecological niches [237]. For example, the use of urban wastewaters favors the selection of *Geobacter* sp. while the use of sediments from a salt plant favors the selection of *Geokalibacter subterraneus* [7, 14]. Therefore, depending on the type of inocula, the diversity of electroactive bacteria might be influenced. Knowing that not all EABs have the same performances, there is a real interest in selecting the most efficient EAB to improve MFC performances ([63, 238].

Currently, several inocula have been used with the objective of improving the MFC performances with real influents such as industrial wastewaters, domestic wastewaters or sediments [11, 80, 239, 240]. However, community analyzes are not always carried out, which makes it impossible to link variations in performances and microbial factors.

In this study, to develop the most efficient microbial consortium to treat industrial wastewater, three different sources of inoculum were investigated. The use of sludge issued from an industrial wastewater treatment plant as inoculum was compared to anaerobic sludge sampled from an urban wastewater treatment plant, as the most commonly used inoculum in BES. Mixtures of several combination of the three inocula (n=6) and the use of the wastewater itself (negative control) were also evaluated. The aim of this study was to understand the impact of diverse inocula (either separated or mixed) according to differences in MFC performances (e.g. maximal current densities 'CD_{max}', coulombic efficiency 'CE', time per batch or organic removal efficiency 'ORE').

5.1 Materials & methods

5.1.1 Industrial wastewater

The first two batches were operated with 90% (v/v) of industrial wastewater (IW) and 10% (v/v) of inoculum. The characteristics of the industrial wastewater are reported in Table 5-1. Only the control conditions were fed with 100% of industrial wastewaters. For the mixture of two inocula, the volumes were 1.4 mL (5%) for each. For conditions with 3 inocula, each represented equally 1/3 of 10% (~0.93 mL). From the third to the fifth batch, only industrial wastewater was added. During the whole experiment the raw wastewater was added, without buffer or amendment.

Table 5-1 Characterization of industrial wastewater used to supply MFCs. COD i.e. chemical oxygen demand.

Chloride (mg.L ⁻¹)	Nitrate (mg.L ⁻¹)	Sulfate (mg.L ⁻¹)	Phosphate (mg.L ⁻¹)	Acetate (mg.L ⁻¹)	Butyrate (mg.L ⁻¹)	Iso-butyrate (mg.L ⁻¹)	COD (mg.L ⁻¹)
488.1	14.5	166.1	161.2	7.42	2.51	2.32	1742

5.1.2 Inocula

Three different inocula were used in this study: i) a sludge from industrial wastewater treatment plant from the facility providing the wastewater used as substrate, termed “I”; ii) an aerobic sludge sampled from the aeration tank of municipal wastewater treatment plant (Narbonne, France) termed “U”; iii) an EAB-enriched culture in acetate-fed air-cathode MFC termed

“EAB”. Mixtures (1:1) of two inocula were made with: “I” and “EAB” called “I_EAB”; “U” and “EAB” called “U_EAB” and a mixture (1:1:1) with the three inocula “I,” U” and “EAB” called “U_I_EAB” has also been put in place.

5.1.3 MFC configuration

A single-chamber, air-cathode MFC was constructed as described previously [26]. The anode electrode (projected surface area = 8 cm²) was made of carbon felt, and the cathode was made of carbon cloth containing a Pt catalyst (0.35 mg/cm², water-facing side; E-TEK). The air-facing side of the cathode was coated with four PTFE diffusion layers as previously described (Cheng et al. 2006a). The electrodes were placed on opposite sides of the cylindrical chamber (4 cm long, 3 cm diameter; 28 mL liquid volume) (Fig. 5-1). Silver wire was used for the connection of the external circuit to the carbon electrodes. MFC test were performed in duplicate.

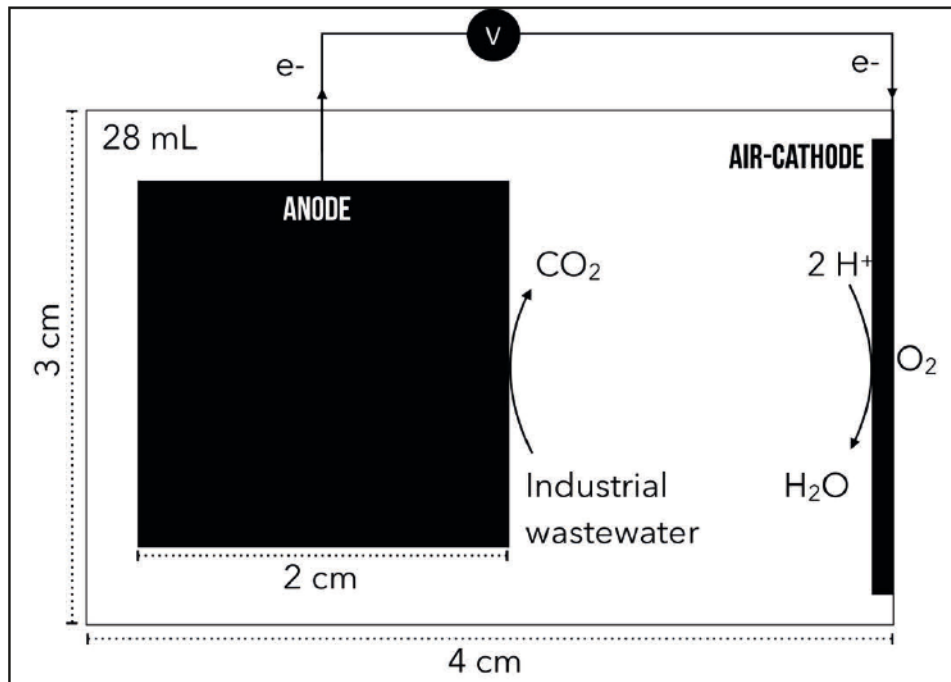


Fig. 5-1 Microbial fuel cell design used in this study.

5.1.4 Microbial fuel cell set up and operation

Fourteen reactors were operated in fed-batch mode at a fixed external resistance of 470Ω and operated in fed-batch mode, for a total of five batches. The voltage across the external resistance (V) was monitored using a 12-bit data acquisition card (Advantech PCI-1711) connected to a personal computer with AddControl software developed in LabWindows CVI (2014) for data acquisition. All reactors were inoculated with different mixtures of the three inocula as well as with unmixed inocula and negative control (without exogenous inoculum), and operated at ambient temperature at 20°C ($\pm 2^{\circ}\text{C}$). After five batch cycles of power generation the MFCs has been stopped and the electroactive biofilm & bulks collected.

5.1.5 Analytical Methods

Concentrations of organic acids were measured by HPLC with a refractive index detector (Waters R410). First, samples were centrifuged at 13,500g for 15 min and then supernatants were filtered with 0.2 μm syringe filter. HPLC analysis was performed at a flow rate of 0.4 mL/min on an Aminex HPX-87, 300 x 7.8 mm (Bio-Rad) column at 35°C with H_2SO_4 (4 mM) as mobile phase. DCO analysis was carried out using commercial kits (Hach, LCK 714, United States) and a spectrophotometer (Hach, DR2800).

5.1.6 Calculations

MFC performance was evaluated according to methods described in 2006 by Logan et al. [29]. The organic removal efficiency (ORE [%]) was calculated from the influent COD (COD_{in}) and effluent COD (COD_{out}) as $\text{ORE} = [\text{COD}_{in} - \text{COD}_{out}] / \text{COD}_{in}$. Current ($I=V/R$) was calculated using the measured voltage (V), the resistance (R). By dividing the maximum current intensity by the anode area in m^{-2} , the maximum current density ($\text{A}\cdot\text{m}^{-2}$) was calculated. Coulombic efficiency (CE) was estimated based on total COD removal ($\text{COD}_{in} - \text{COD}_{out}$) and the measured current using: 1 g of COD = 0.125 mol of electron. T^{80} (Time Per Batch – 80%) was defined as the time (in hour) needed to remove 80% of the initial COD.

5.1.7 Microbial community analysis

For each batch, the planktonic part was sampled after inoculation as the starting point, constituting the inoculum samples. At the end of each batch, the planktonic part was collected and constituted the bulk samples and the anodic biofilm was harvested with a blade. These three types of samples (Inocula, bulks and biofilms) were centrifuged at 13,500g for 15 min and the pellet was stored at -20°C prior to microbial community analyses. DNA extraction was carried out with QIAamp fast DNA stool mini kit in accordance with the manufacturer's instruction (Qiagen, Hilden, Germany). DNA extraction was confirmed using Infinited 200 PRO Nanoquant (Tecan Group Ltd., Männedorf, Switzerland). Amplicons from the V3 to V4 regions of 16S rRNA genes were amplified with bacterial forward 343F 5'-TACGGRAGGCAGCAG-3'; and reverse 784R 5'-TACCAGGGTATCTAATCC-3'; primers. Adapters were added for multiplexing samples during the second amplification step of the sequencing. The PCR mixtures (50 µL) contained 0.5 U of Pfu Turbo DNA polymerase (Stratagene) with its corresponding buffer, 0.5 mM of each primer, 200 mM of each dNTP and 10 ng of genomic DNA. Reaction were carried out in a Mastercycler thermal cycler (Eppendorf) as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min. The size and amount of PCR products were defined using a Bioanalyser 2100 (Agilent). The community composition was evaluated using the MiSeq v3 chemistry (Illumina) with 2 x 300 bp paired-end reads at the Genotoul platform (www.genotoul.fr). Sequence were retrieved after demultiplexing, cleaning, and affiliating using Mothur [165]. All sequences were submitted to Genbank under accession numbers MH461134-MH463048.

5.1.8 Quantitative PCR (qPCR)

PCRs were prepared using 96-well real time PCR plates (Eppendorf, Hamburg, Germany) and Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). After, 6.5 μ L of Express qPCR supermix with premixed ROX (Invitrogen, France), 2 μ L of DNA extract with three appropriate dilutions, 100 nM forward primer F338-354 (5'-ACTCCTACGG GAGGC AG-3'), 250 nM reverse primers R805-785 (5'-GACTA CCAGG GTATC TAATC C-3'), 50 nM TaqMan probe and water were added to obtain a final volume of 12.5 μ L for all analyses. A first incubation of 2 min at 95°C followed by 40 cycles of denaturation (95°C, 7 s; 60°C, 25 s) was performed. From each assay, one standard curve was generated by using tenfold dilution in sterilized water (Aguettant Laboratory, Lyon, France) of a target plasmid (Eurofins Genomics, Germany). The initial DNA concentrations were quantified using the Infinite 200 PRO NanoQuant (Tecan, France).

5.1.9 Statistical analysis

All statistical analyses were carried out in R version 3.2.3. The differences between conditions were tested with one-way ANOVA followed by Tuckey's HSD post-hoc test with a statistical significance (P -value) < 0.05 after verifying the normality of the variables by the Shapiro-Wilk test and the homoscedasticity with the Bartlett's test. PCoA ordination based on Weighted-

UniFrac distances was used to represent inter-samples distances with phyloseq package [166]. ARB software was used to calculate the phylogenetic distances between OTUs with SILVA 123 as reference tree [241]. The Monte Carlo simulation was used to test the difference between microbial composition according to the inoculation strategy with *P*-value of 0.001. Alpha diversity index was calculated with the Microbiome package. Co-occurrence network was constructed with the igraph package. Newman-Girvan algorithm was used to identify subcommunity [242]. Betweenness Centrality and Eigenvalue Centrality was calculated with the MicrobiomeSeq package. *Spearman* coefficient was used as correlation index for calculated correlation between OTUs with a significant threshold of 0.6 and *P*value < 0.01.

5.2 Results and discussion

5.2.1 MFC performances

MFCs were run in successive fed batch mode until reproducible current production were obtained. To compare the effectiveness of the different inoculation conditions, four performance indices were assessed: (1) the maximum current densities (CD_{max}); (2) the coulombic efficiency (CE); (3) the time (in hour) needed to remove 80% of the initial COD (T^{80} : Time per Batch) and (4) the organic removal efficiency (ORE). The values obtained for the fifth batch are reported in Table 5-2. The T^{80} values range from 12.48±0.07 hours for condition U_I_EAB to 15.02±1.73 hours for condition NC. The ORE values range from 77.03±9.58% for condition I to 63.42±9.49% for condition NC which is consistent with similar

studies [243, 244]. No significant difference was observed between all conditions for the T^{80} and ORE indices. CD_{max} values showed significant differences between conditions. The I condition had a significantly higher average ($0.65 \pm 0.04 \text{ A.m}^{-2}$) compared to the U_I_EAB and NC (Negative control) conditions with $0.195 \pm 0.16 \text{ A.m}^{-2}$ and $0.235 \pm 0.05 \text{ A.m}^{-2}$ respectively. However, compared to other similar studies, these CD_{max} were relatively low [142, 153]. Concerning coulombic efficiency, the I condition had the highest average with $70.2 \pm 18.36\%$, significantly higher than the U_I_EAB condition with a coulombic efficiency of $13.86 \pm 10.90\%$. Compared to similar studies, coulombic efficiencies were relatively high which suggests little electron leakage [142, 153].

As described above, inoculation strategies had a significant impact on two performance indices: CD_{max} and CE. Only the I strategy outperforms the negative control and the mixture of the three exogenous inocula. This means that the indigenous microbial community (in the industrial wastewater) is not suitable as an inoculum for MFC treatment, and mixing the three exogenous inocula, a potential source of high biological diversity, is ineffective for improving MFC performances. The standard deviations of the ORE were smaller (0.47-9.98) than those of the CE (4.82-21.16). Thus, for the same duplicate, the decrease in COD was close (i.e. low standard deviation) but the efficiency of electron recovery at the anode was variable (i.e. large standard deviation). This observation suggests that significant microbial competition between bacteria capable of using the anode as an electron terminal acceptor (i.e. EAB) and other respiratory modes based on molecules that were present in industrial wastewaters such as nitrates or sulfate, probably occurred (Table 5-1).

Table 5-2 Performances indices for the conditions obtained for the batch V. Values sharing the same exponent letter are not significantly different. 'ns' means no significant difference.

Conditions	Inoculum/a	CD_{max} (A.m ⁻²)	CE (%)	T^{80} (Hours)	ORE (%)
EAB	EAB	0.365±0.05 ^(abc)	41.47±6.16 ^(ab)	13.90±1.59 ^(ns)	66.46±0.47 ^(ns)
U	U	0.365±0.05 ^(abc)	28.09±4.82 ^(ab)	13.27±0.51 ^(ns)	69.28±4.63 ^(ns)
I	I	0.65±0.04 ^(a)	70.02±18.36 ^(a)	13.47±0.06 ^(ns)	77.03±9.58 ^(ns)
U_EAB	U+EAB	0.325±0.13 ^(abc)	22.52±12.39 ^(ab)	13.24±0.16 ^(ns)	73.30±5.43 ^(ns)
I_EAB	I+EAB	0.495±0.02 ^(abc)	46.33±21.16 ^(ab)	12.78±0.74 ^(ns)	69.05±9.98 ^(ns)
U_I_EAB	U+I+EAB	0.195±0.16 ^(b)	13.86±10.90 ^(b)	12.48±0.07 ^(ns)	65.78±15.1 ^(ns)
NC	-	0.235±0.05 ^(c)	14.57±15.02 ^(ab)	15.02±1.73 ^(ns)	63.42±9.49 ^(ns)

5.2.2 Microbial analysis

To better understand the link between the inoculation strategy and the observed variations in performances, analysis of microbial communities was performed.

5.2.2.1 Microbial diversity analysis

5.2.2.1.1 Diversity of planktonic communities

To determine whether there were differences in planktonic community structures, a PCoA analysis based on Weighted-UniFrac distances was carried out, a graphical output is shown in Fig. 5-2. Weighted-UniFrac distances incorporates information on the relative relatedness of the community members by incorporating species accounts at each sample and the phylogenetic distances between observed organisms in the computation [245]. The greater the Weighted-UniFrac distances, the more the bacterial communities diverge in terms of phylogeny and abundances and inversely. The magnitude of the eigenvalue of each axis indicates the amount of the variation captured in that axis. Axis.1 represents 33.6% of the variance and axis.2 29.7%. Within the same duplicate, conditions U_I_EAB and EAB have the smallest Weighted-UniFrac distances with 0.12 and 0.05 respectively which indicates similar bacterial communities [246]. The I condition had the highest intra-sample distance (0.22) which indicates dissimilar bacterial communities. Except for condition I, the bulks of all other conditions are clustered on the right of the axis.1. Interestingly, the negative control, without exogenous inoculum, was close to conditions U_I_EAB, U and sample I_EAB_2. The sample I_1 was the most different from the others.

Strategies U_I_EAB, U or I_EAB were closer to the NC condition, i.e. representing endogenous consortia from the industrial wastewater, when compared to the I condition. The inocula were added at the beginning of the first two batches. Then, only industrial wastewater was fed on the three consecutive batches. Industrial wastewater provided a lot of diversity which is not constant but varies according to the stock of wastewater (Industrial WW_1 & IndustrialWW_2 Fig. 5-6.a). So, according to the PCoA ordination, the initial inoculation strategy had an impact on the structure of the planktonic communities, despite the addition at

each batch of the bacteria endogenous from industrial wastewater. Consequently, it could be that at each batch, the consortium present in the industrial wastewater and those remaining in the MFC (i.e. on the reactor walls, the anode and the air-cathode) were competing for the fastest access to substrates. Depending on the bacterial consortia previously formed by the different inoculation strategies, some may be more competitive for access to substrates than others. For example, those formed by inoculum I seem more competitive than those formed by inocula U_I_EAB and U against consortia present in industrial wastewater. This analysis therefore shows two probable evolutions of planktonic communities according to the inoculation strategy: (1) convergent evolution (i.e. uncompetitive consortia) towards negative control for conditions U_I_EAB, U or I_EAB and (2) divergent evolution (i.e. competitive consortia) towards a specific structure for conditions I, EAB and I_EAB.

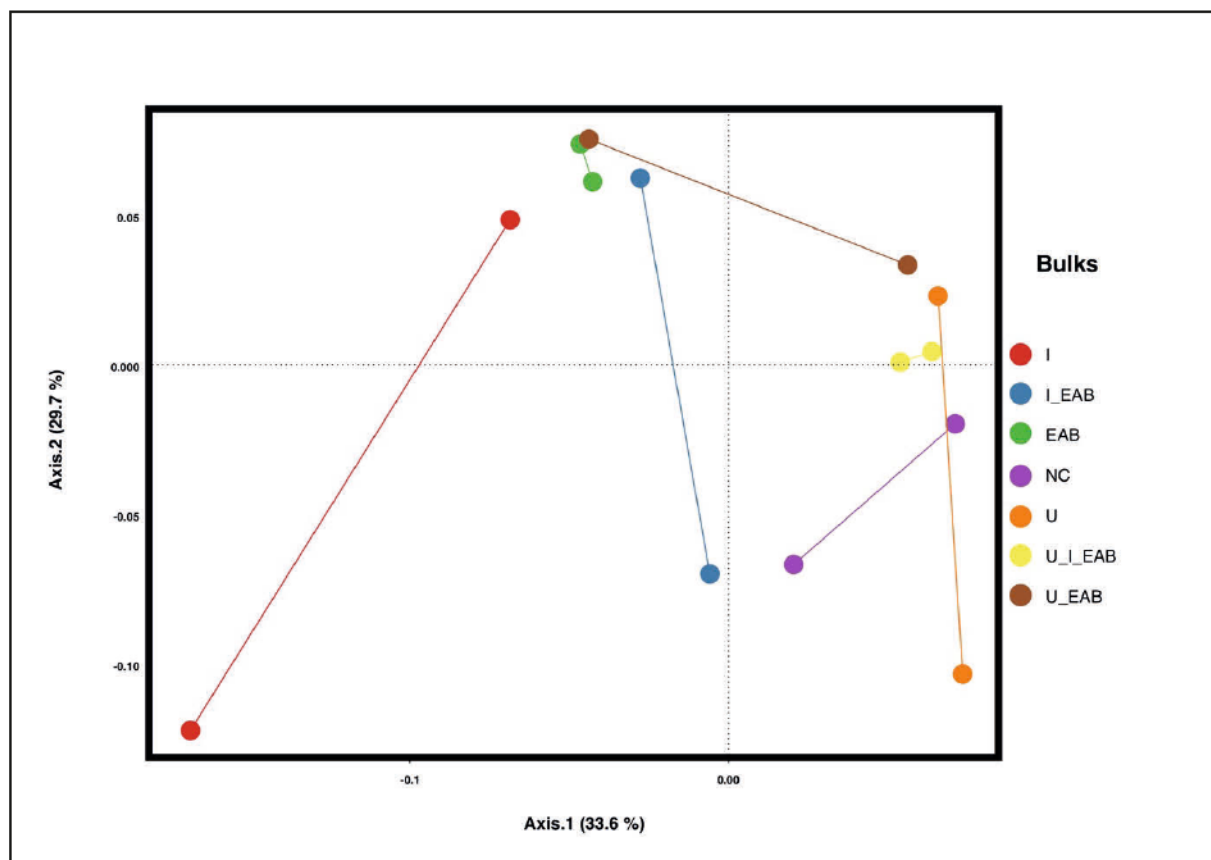


Fig. 5-2 Principal coordinate analysis (PCoA) based on weighted-UniFrac distance matrix calculated from the planktonic taxa showing the distribution patterns of all samples.

To determine whether alpha diversity differences can be observed as a function of conditions, three indices were calculated: (1) The specific richness or Richness, i.e. the number of different species (or OTUs) in a specific habitat, allows to determine if the number of OTUs is different or not according to the inoculation conditions; (2) the Shannon index, which takes into account of the specific richness and the distribution of individuals within these species (specific evenness); (3) the Pielou's evenness or species evenness, which evaluates the similarity of frequencies of the different species making up a sample. Species evenness allows to determine if the proportion of OTUs was different or not depending on the inoculation conditions. Pielou's

evenness varied from 0 i.e. an unbalanced ecosystem to 1 i.e. a balanced ecosystem (equal frequencies between all species) (Fig. 5-3).

The highest richness value was found in sample U_2 and the lowest in NC_1 with 140 and 317 OTUs respectively. The greatest difference in specific richness within the same condition was observed for inoculum U with varies from 162 OTUs for samples U_1 to 317 OTUs for U_2. The smallest difference in specific richness within the same condition was observed for inoculum I with only 5 species difference. The I and I_EAB samples had a significantly higher richness than the EAB condition. Interestingly, the condition where the three inocula were mixed (U_I_EAB) did not have higher number of OTUs compared to the other conditions. The evenness between OTUs varied from 0.51 (I_EAB_1) to 0.60 (U_2) which means that all samples had a balanced ecosystem (no dominance of one species). The NC condition had a higher evenness than I, EAB and U_EAB conditions. However, concerning Shannon's diversity, no significant difference was observed. Indeed, only the industrial wastewater was added on the last three batches. This wastewater was composed of microorganisms which may explain why the initial inoculation strategies had no effect on Shannon's diversity.

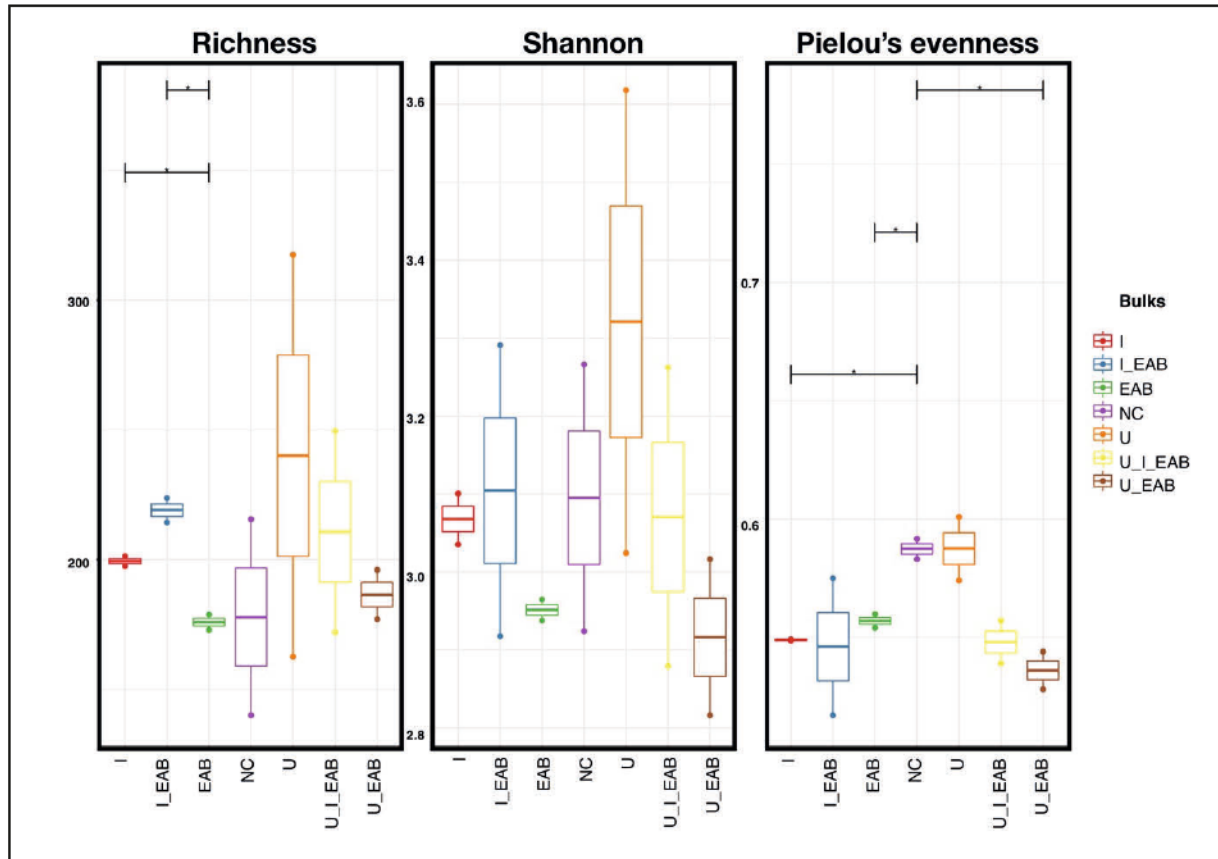


Fig. 5-3 Alpha diversity of planktonic conditions according to three indices: richness, Shannon and Pielou's evenness. The horizontal bars show the significant differences: * p value < .05.

5.2.2.1.2 Diversity of anodic communities

To analyze the differences in the community structures of the anodic communities of the different conditions, a PCoA ordination based on the Weighted-UniFrac distances was performed, it is shown in Fig. 5-4: Axis.1 represents 40.8% of the variance and axis.2 17.1%. Some inoculation conditions had more difference in community structure than others. For example, the Weighted-UniFrac distances was the lowest for condition I (0.28) and the highest for condition U_I_EAB (0.51). There was therefore structural variability within the same

inoculation strategy, particularly for the I condition. The conditions U, U_EAB and NC were all clustered at the top of the ordination suggesting anodic communities close in terms of abundance and phylogenetic diversity. The other conditions, I, U_I_EAB, I_EAB and EAB had short distance within the same duplicate on the PCoA analyzes, suggesting a bacterial structure specific to an inoculation condition. The bacterial structure can therefore either be stable (small distance between duplicates) or variable (high distance between duplicates). It would be interesting to determine the ecological mechanisms allowing to have a stable microbial structure, i.e. predictable from a given inoculum in the case of a larger scale use. So, the PCoA analysis shows two types of anodic consortia evolution: (1) a structure close within the same duplicate concerning I, I_EAB, EAB and U_I_EAB) conditions; (2) on the other hand a structure close to the negative control condition (U & U_EAB).

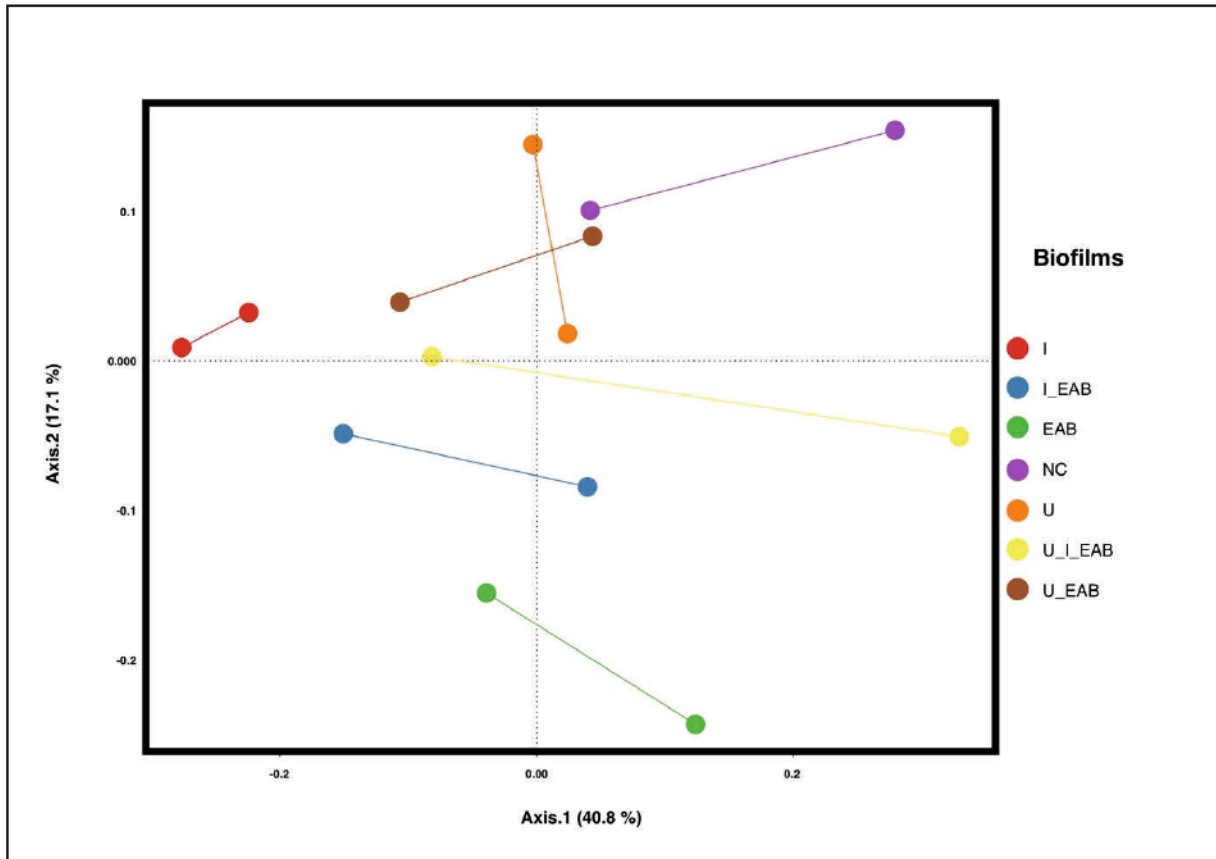


Fig. 5-4 Principal coordinate analysis (PCoA) based on weighted-UniFrac distance matrix calculated from the taxa of the biofilms showing the distribution patterns of all samples.

As for bulk samples, three diversity indices were calculated (Richness, Shannon & Pielou's evenness) (Fig. 5-5). Richness varies from 194 OTUs (I_2) to 577 OTUs (U_2). Condition U had a significantly higher richness than conditions EAB and I. Condition U_EAB had a significantly higher richness than condition EAB. Concerning the Pielou's evenness, no significant difference was observed between conditions. This index varies from 0.44 (I_2) to 0.75 (NC_2) indicating a high equitability and no dominance of one or few species compared to others electroactive biofilms [213]. As for the evenness, the Shannon index was not significantly different between conditions. This index varies from 2.36 (I_2) to 4.5 (NC_2) but

it was observed no significant difference between conditions. Anodic diversity was high compared to other electroactive ecosystems [213]. This high diversity could be due to the material used, carbon felt, which can inhabit planktonic species by storing the liquid medium, unlike other materials such as carbon plates [247].

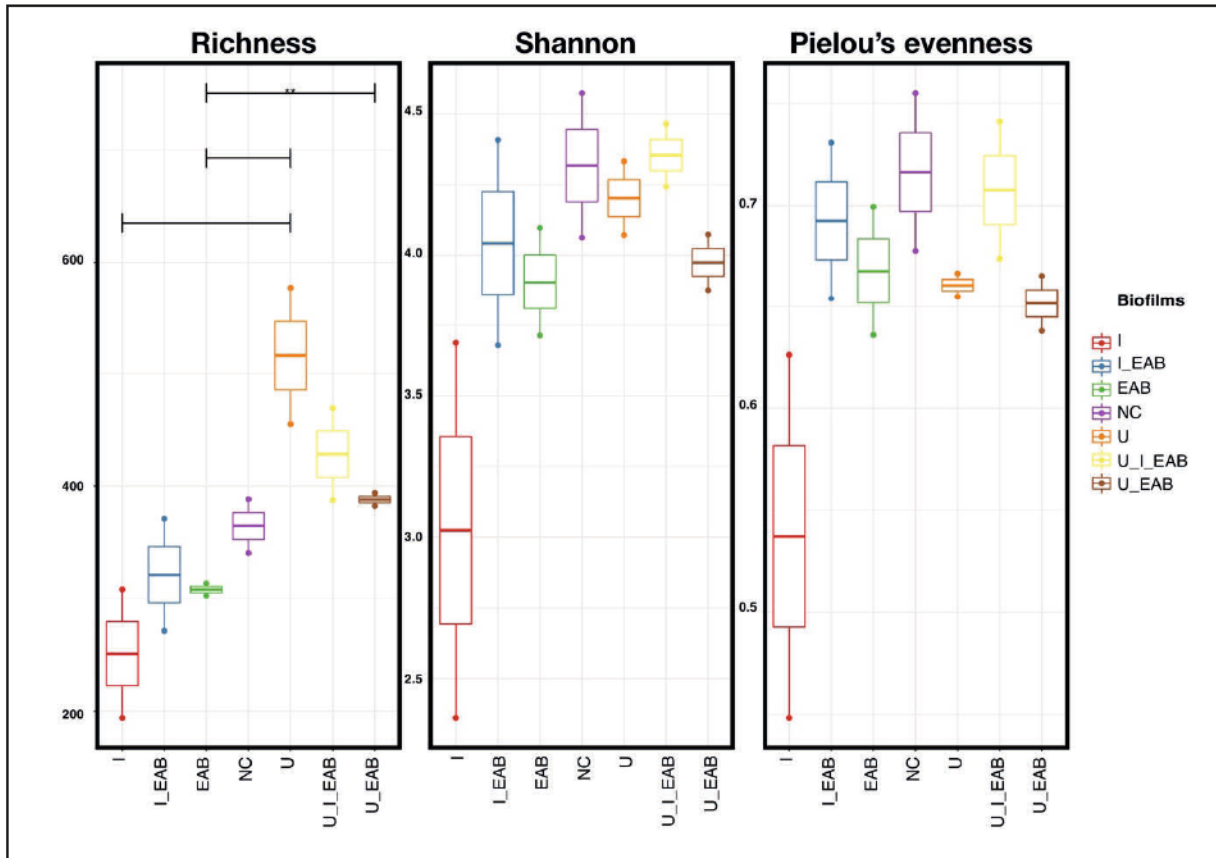


Fig. 5-5 Alpha diversity of anodic conditions according to three indices: richness, Shannon and Pielou's evenness. The horizontal bars show the significant differences: * p value < .05, ** p value < .01.

5.2.2.2 Composition of microbial communities

5.2.2.2.1 *Composition of planktonic communities*

Fig. 5-3 shows the distribution of the taxa found in the bulk phase. On the Fig. 5-3.a the dominant bacteria, i.e. more than 3% in at least one sample, are represented at the genus level with the rest of the diversity, i.e. less than 3% in all samples, grouped in the 'Others' category. The wastewater was analyzed before use. Industrial_WW_1 was used to feed the first two batches and Industrial_WW_2 for the last three batches. Whatever the conditions, the low abundant species represented more than 50% of the total bacteria found in the bulk. On the Fig. 5-3.b is represented only the dominant genera. The genera *Imtechella* sp. and *Owenweeksia* sp. were predominant in the bulk phase of all the conditions.

The three major genera were *Owenweeksia* sp., *Imtechella* sp. and *Desulfomicrobium* sp. whatever the condition. *Owenweeksia* sp. is a strict anaerobic heterotrophic genus capable of growing on yeast extract and peptone [248]. This genus was negatively correlated to ORE (-0.79). So the more abundant *Owenweeksia* sp. is, the less COD decreases during the batch which is surprising in view of his metabolic abilities (Table 5-3). As previous determined, *Imtechella* sp. is a strict aerobic and capable of oxidizing carbohydrates such as fructose, xylose and mannose [249]. As the MFCs are re-feed at each batch, it is possible that oxygen could enter the liquid medium (opening of the reactors to the ambient air). This could explain the growth of *Imtechella* sp. Using sulfate as terminal electron acceptor, *Desulfomicrobium* sp. is strictly anaerobic [250]. This sulfato-reducing genus could use the sulfate that was present in industrial wastewater (166.1 mg.L^{-1}) as electron acceptor with lactate, pyruvate, ethanol formate and H_2 as electron donors. The presence of these two respiratory modes, based on oxygen & sulfate,

indicates possible losses of electrons and thus a decrease in coulombic efficiency, although their abundances were not significantly correlated with CE.

The genus *Anaerophaga* sp. was highly negatively correlated to ORE (-0.79). This strict anaerobic bacterium is capable of fermenting hexoses and pentose to acetate succinate and propionate. Propionate being recalcitrant in MFC, its production could have a negative impact on ORE [184, 251]. However, this metabolite was not detected in HPLC analysis as previously observed in Table 5-1. *Microbacter* sp. was also highly negatively correlated to ORE (-0.76). This taxon is known to generate propionate from carbohydrates, which could explain the negative link with ORE [252]. *Comamonas* sp. was negatively related to CD_{max} (-0.63) and ORE (-0.69). This bacterium is capable of reducing nitrates, who were present at 14.5 mg. L⁻¹ in industrial wastewater, with organic acids i.e. acetate, lactate, fumarate and amino acids as electron donors, indicating that it may be competing with anodic electron recovering [253]. *Sulfurospirillum* sp. was positively correlated to CD_{max} (0.67) on the one hand and negatively to ORE (-0.68) on the other. This bacterium can oxidize lactate and H₂ using many electron terminal acceptors (Selenate, Arsenate, Thiosulfate, S⁰, Fe(III), MnO₂) [254]. Its ability to reduce Fe(III), requiring an exo-electrogen mechanism may explain its link with CD_{max} (e.g. reduction of electronic shuttles). The negative link between *Sulfurospirillum* sp. and the ORE is not clear with current knowledge. *Chlorobium* sp. was positively correlated with CD_{max} ($r = 0.70$) and CE ($r = 0.65$) and *Sulfurovum* sp. only with CE ($r = 0.66$). These two genera are capable of oxidizing thiosulfate as electron donor [255, 256]. In view of current knowledge, their links with CD_{max} and CE is not yet understood.

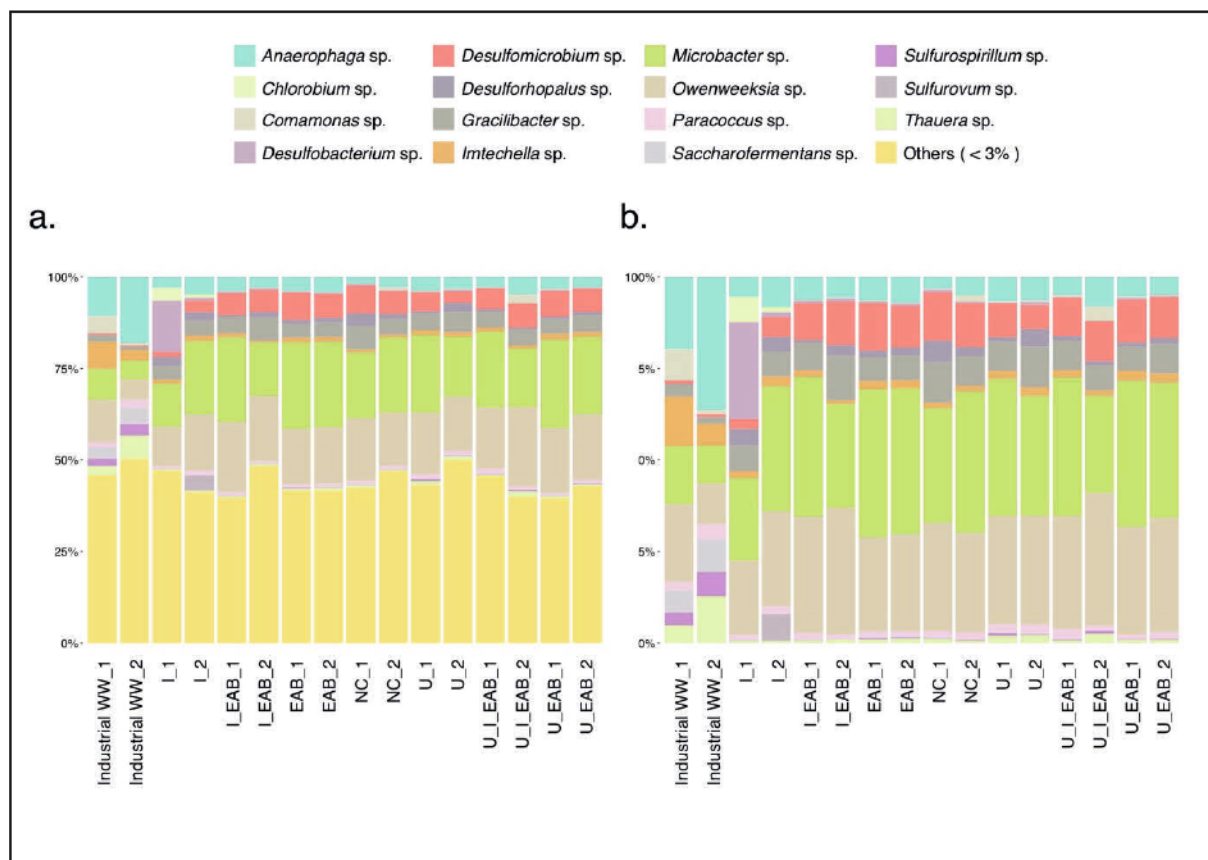


Fig. 5-6 Relative tax abundances at Genus level of all planktonic samples. (a). Representation of the dominant genera with the rest of the diversity classified in the 'Others' categories. (b) Representation of the dominant genera.

Table 5-3 Correlation of major taxa of planktonic samples with performance indices. * p value < .05, ** p value < .01, *** p value < .001.

Genus	CD _{max}	CE	ORE
<i>Anaerophaga</i> sp.	-0.20	0.12	-0.79**
<i>Chlorobium</i> sp.	0.70**	0.65*	0.48
<i>Comamonas</i> sp.	-0.63*	-0.39	-0.69**
<i>Desulfobacterium</i> sp.	0.51	0.39	0.50
<i>Desulforhopalus</i> sp.	-0.02	0.00	-0.17

<i>Gracilibacter</i> sp.	-0.31	-0.19	-0.51
<i>Imtechella</i> sp.	-0.08	0.14	-0.50
<i>Microbacter</i> sp.	-0.35	0.02	-0.76**
<i>Owenweeksia</i> sp.	-0.50	-0.23	-0.79***
<i>Paracoccus</i> sp.	-0.23	0.01	-0.60
<i>Saccharofermentans</i> sp.	0.15	-0.21	0.23
<i>Sulfurospirillum</i> sp.	0.67**	-0.49	-0.60*
<i>Sulfurovum</i> sp.	0.53	0.66**	0.03
<i>Thaurea</i> sp.	-0.49	0.43	-0.44

5.2.2.2.2 Composition of anodic communities

Relative abundance of the dominant genera, i.e. more than 3% in at least one sample, found in the biofilm communities can be observed Fig. 5-7.b. Fig. 5-7.a, shows the minority OTUs, i.e. less than 3% in all samples, clustered in the 'Others' categories. Species present in low abundance represent more than 50% of total diversity for all the samples. Within the majority taxa, Fig. 5-7.b, there was a dominance of the genera *Geobacter* sp., *Owenweeksia* sp. and *Thiobacillus* sp..

Geobacter sp. was mainly present in I_1 sample. The species belonging to this genus are known to be electroactive, i.e. capable of using an electrode as an electron terminal acceptor [61]. *Geobacter* sp. are capable of oxidizing short-chain acids such as acetate, butyrate, propionate and lactate as well as simpler molecules such as hydrogen or formate [97]. This genus was

positively correlated to CD_{max} and CE which seems in agreement with its electroactivity ability (Table 5-4). It was probably a key player in the anodic communities, allowing to explain the high performances of the I condition, where this taxon was predominant.

Owenweeksia sp. was mainly present in the U_EAB condition. This genus has been described above because it was also present in the planktonic community where it was significantly anti-correlated with ORE. In biofilms, the opposite trend was observed because this genus was positively correlated with ORE (+0.59). Its metabolism based on the use of yeast extract and peptone in anaerobes could contribute significantly to the reduction of COD, explaining this relationship with ORE.

Thiobacillus sp. uses S° as electron donor and sulfate as electron terminal acceptor [257]. Interestingly, this taxon was not abundant in the planktonic community, suggesting a preference for the anode as an ecological niche. This genus has been characterized as electroactive, being active on a bio-cathode [142]. It seems that this sulfo-oxidizing bacterium uses the anode as an electron acceptor, explaining its abundance in biofilm communities in contrast to the liquid medium. The fact that this taxon was positively correlated with CD_{max} seems coherent in view of these electroactivity capabilities.

The genera *Lentimicrobium* sp. and *Anaerophaga* sp. were significantly correlated to CD_{max} and EC. These taxa are capable of using many carbohydrates (e.g. glucose, ribose, sucrose, maltose, mannose, pyruvate and starch) [217, 251]. They could contribute significantly to fermentation processes by producing short-chain acids which are preferably used by electroactive bacteria (e.g. *Geobacter* sp.).

Paraclostridium sp. and *Pseudomonas* sp. were negatively correlated to CD_{max} and ORE. These two taxa are capable of oxidizing many carbohydrates [91, 190]. Their negative contribution to CD_{max} remains uncharacterized.

Thaurea sp. was negatively correlated with CD_{max} . This taxon has been previously characterized within electroactive biofilms and is probably electroactive [258]. It was the dominant bacteria on a bio-cathode of a MFC [259]. Its electron donors are acetate, propionate and ethanol (that was present in industrial wastewater; Table 5-1) with nitrate as terminal electron acceptor. Therefore, this bacterium could compete with electroactive bacteria for the substrates, explaining the negative relationship with CD_{max} .

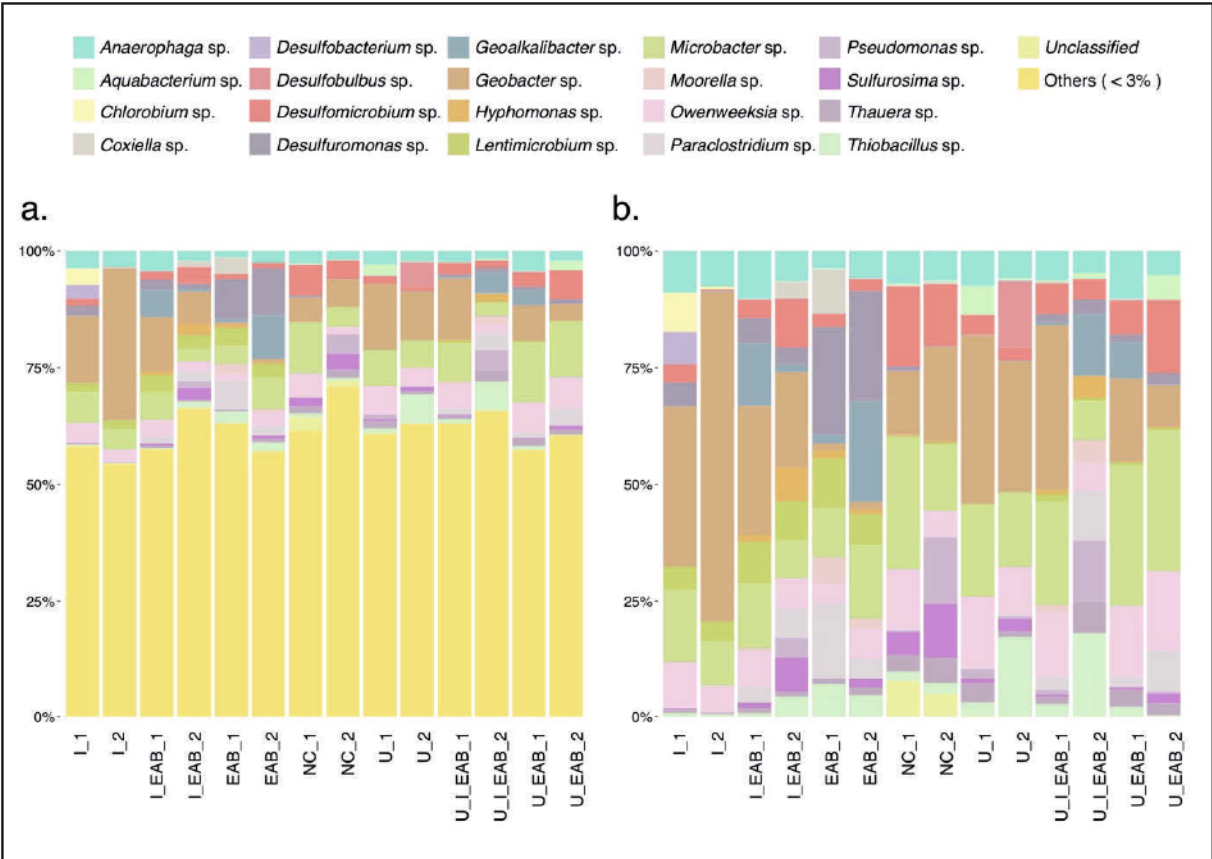


Fig. 5-7 Relative taxa abundances at Genus level of all anodic samples. (a). Representation of the dominant genera with the rest of the diversity classified in the 'Others' categories. (b) Representation of the dominant genera.

Table 5-4 Correlation of major taxa of anodic samples with performance indices. * p value < .05, ** p value < .01, *** p value < .001.

Genus	CD _{max}	CE	ORE
<i>Geobacter</i> sp.	.67**	.73**	.31
<i>Microbacter</i> sp.	.08	-0.03	.43
<i>Owenweeksia</i> sp.	.19	.006	.59*
<i>Desulfomicrobium</i> sp.	-.34	-.34	.01
<i>Anaerophaga</i> sp.	.60*	0.59*	.41
<i>Desulfuromonas</i> sp.	.14	.13	.01
<i>Thiobacillus</i> sp.	-.54*	-.48	-.51
<i>Geoalkalibacter</i> sp.	-.06	-.01	-.30
<i>Paraclostridium</i> sp.	-.58*	-.26	-.64*
<i>Lentimicrobium</i> sp.	.75**	.78***	.23
<i>Thauera</i> sp.	-.67**	-.47	-.43
<i>Sulfurosima</i> sp.	-.31	-.11	-.32
<i>Pseudomonas</i> sp.	-.58*	-.26	-.64*
<i>Desulfobulbus</i> sp.	.04	-.06	-.11
<i>Hyphomonas</i> sp.	-.22	-.31	-.27
<i>Moorella</i> sp.	-.46	-.32	-.44
<i>Coxiella</i> sp.	-.006	.079	-.004
<i>Aquabacterium</i> sp.	-.37	-.45	.01
<i>Chlorobium</i> sp.	.46	.33	.53*
<i>Desulfobacterium</i> sp.	.44	.30	.53*

5.2.2.2.2.1 Identification of anodic key species

As previously observed, electroactive biofilms were mainly composed of low abundance genera. To identify within the numerous taxa, the key players, a co-occurrence analysis was carried out (Fig. 5-8). This analysis generated a microbial community network representing the co-occurring genera in all electroactive biofilms. Lines represent significant positive correlation (threshold > 0.6 , $Pvalue < 0.01$). Taxa were clustered into nine subcommunities according to the Newman-Girvan clustering, each are represented by one color [242]. For each subcommunity, two indices have been calculated: Betweenness Centrality (BC) & Eigenvalue Centrality (EC) (Fig. 5-8.a). These indices were calculated for each node representing a bacterial genus (symbolized by circles in Fig. 5-8) and then applied to each subcommunity by averaging the values of each nodes belonging to it. BC is a measure of the importance of one genus, calculated from the number of times a genus is used as a crossing point to connect two other genera along the shortest path. The higher the BC, the more influence taxa have on the network and vice versa. Within biofilms, no subcommunity had a different BC from the others, which could imply that there is no central subcommunity to the network (Fig. 5-8.a).

Eigenvalue Centrality (EC) is also a measure of the importance of one genus calculated from this connectivity with other genera. More one genus is connected to others that were themselves very connected, more the EC is and vice versa. In biofilms, subcommunity 2 had a significantly

higher EC than the others, meaning that this subcommunity was the most connected compared to the others.

Fig. 5-8.b shows the co-occurrence network. The colors of each circle, i.e. a genus, corresponds to the belonging subcommunity, the lines represent the positive and significant correlations, and the size of the circle is proportional to the degree of connectivity (i.e., the number of links).

The key taxa of each subcommunity, selected because it had the most link among the genera of the subcommunity, are represented on the network by indicating the genus name (Fig. 5-8.b).

The latter probably had an important role in electroactive biofilms by interacting with many other genera. Subcommunity 1 was represented by *Paludibaculum* sp., a facultative anaerobic bacterium capable of fermenting many carbohydrates i.e. glucose, fructose, galactose [260].

These fermentative products, i.e. acetate, propionate, H₂, CO₂ can be used as electron donors by many bacteria such as EAB this could explain the links with several other taxa.

Subcommunity 2 was represented by *Gemmatimonas* sp., capable of using yeast extract, succinate and acetate as electron donors and using O₂ as electron acceptor [261]. Interestingly,

this taxon was among the group with the highest EC, meaning it had a strong connection with other highly connected genera in the network. Subcommunity 3 was represented by

Comamonas sp., capable of reducing nitrate with valerate, acetate, lactate and 3-Hydroxybutyrate [262]. Subcommunity 4 was represented by *Paracoccus* sp. This aerobic

taxon grows on a wide range of carbon sources (e.g. glucose, fructose, galactose, mannose, arabinose) [263]. Subcommunity 5 was represented by *Desulforhopalus* sp., a sulfate-reducing

bacterium isolated at 10°C from reduce mud. This bacterium use propionate, lactate and alcohols as electron donors and carbon sources by producing acetate, an electron donor rapidly

oxidized by EABs. *Desulforhopalus* sp. grew heterotrophically only with hydrogen as electron donor which can decrease the CE in MFC by storing electrons as biomass instead of being transferred to the anode [264]. Subcommunity 6 was represented by *Acidaminobacter* sp., an L-glutamate-fermenting bacterium. This genus grew slowly on glutamate (μ_{\max} 0.06 h⁻¹) and formed acetate, CO₂, hydrogen and minor amounts of propionate [265]. Subcommunity 7 was represented by *Marinilabilia* sp., a facultative anaerobic genus capable of fermenting melibiose and sorbitol [266]. *Anaerophaga* sp., was the key bacteria of the subcommunity 8. This genus, strictly anaerobic, is capable of using 4-aminobutyrate and 4-hydroxybutyrate as growth substrates [251]. Subcommunity 9 was represented by *Thiothrix* sp., a filamentous sulfur-oxidizing genus. The primary natural habitat of *Thiothrix* sp. is sulfide-containing flowing water where its ability to attach objects gives an advantage compared to other non filamentous bacteria [267].

A correlation analysis between the key taxa of the subcommunities and the performance indices has been performed to determine whether a subcommunity was related to performance indices. The results are presented in Table 5-5. Among the eight key taxa, only *Anaerophaga* sp., i.e. subcommunity 8, was positively correlated with two performance indices: *CD_{max}* and ORE. As previously described, this genus is capable of produce mainly acetate, H₂ and ammonia from 4-aminobutyrate and 4-hydroxybutyrate as electron donors. Acetate and H₂ are preferably oxidized by electroactive bacteria such as *Geobacter* sp. which could explain the relationship of this fermentative taxon with *CD_{max}* [60]. Despite the high connectivity of the other key genus, they were not significantly correlated to the performance indices (Table 5-5).

In conclusion, the co-occurrence analysis identified nine subcommunities within electroactive biofilms. Of these one had high levels of connectivity (subcommunity 2). Interestingly, only the key bacteria of subcommunity 8 was positively correlated with the CD_{max} and ORE. Therefore, from this analysis, it appears that the key player was not in a high connectivity network, which could indicate short trophic pathways between fermenter and electroactive bacteria.

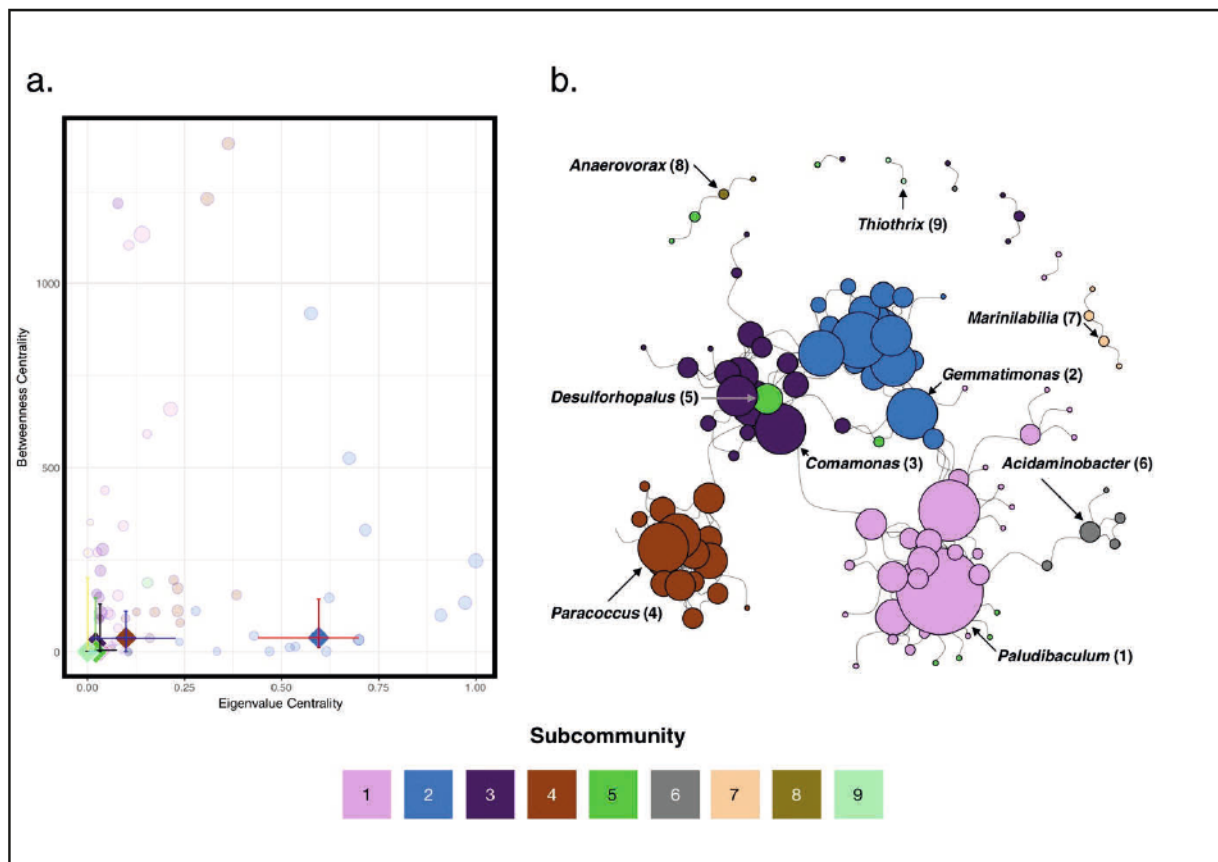


Fig. 5-8 Network analysis showing (A). Relation between Betweenness Centrality (BC) and Eigenvector Centrality (EC). The points represent the position of each taxa corresponds to the calculated BC and EC values. and (B) Co-occurrence network of biofilms genus. Lines represents significant positive correlation (threshold > 0.6 , $Pvalue < 0.01$) and each color represents one specific subcommunity. The colors of each node i.e. circle representing one taxa

corresponds to the belonging subcommunity. The size of the nodes is proportional to its total degree of connectivity.

Table 5-5 Correlation with key genus belonging to one sub community within electroactive biofilms and performance indices.

Sub community	Key Genus	CD _{max}	CE	ORE
1	<i>Paludibaculum</i> sp.	-.32	-.25	-.28
2	<i>Gemmatimonas</i> sp.	-.09	-.13	-.04
3	<i>Comamonas</i> sp.	-.35	-.48	-.09
4	<i>Paracoccus</i> sp.	-.08	-.18	-.08
5	<i>Desulforhopalus</i> sp.	.36	.31	.037
6	<i>Acidaminobacter</i> sp.	.15	.29	.22
7	<i>Marinilabilia</i> sp.	.23	.25	-.15
8	<i>Anaerovorax</i> sp.	.68**	.48	.68**
9	<i>Thiothrix</i> sp.	.05	-.15	-.12

Conclusion

This study investigated the impact of initial inoculation on the performance of industrial wastewater treatment by MFCs reactors with the objective of developing the most effective microbial consortium to treat industrial wastewater. The microbial community developed from the inoculum which showed the best performances during MFC operation was I compared to the other conditions. To understand this performance improvement, a detailed community

analysis was performed on planktonic and anodic bacteria. The planktonic community structure of the I condition was different compared to the negative control. It seems that the consortia of this inoculum were competitive with those present in the wastewater for access to the substrate. *Chlorobium* sp. and *Sulfurovum* sp. were positively correlated with performance indices (CE for both and CD_{max} for *Chlorobium* sp.) suggesting that both bacteria contributed significantly to performance improvement.

Concerning anodic biofilms, the community structure was diverse depending on the inoculum used except for U, U_EAB and EAB conditions closed to the negative control which shows two different evolutions depending on the inoculation condition: (1) divergence of communities towards a specific structure or (2) convergence of communities towards negative control. Anodic communities had a highly condition-dependent structure. The diversity analysis showed a clear trend that the I condition had more resilient consortia compared to those present in industrial wastewater. *Geobacter* sp. was predominant in the most efficient condition (i.e. I) and positively correlated to CD_{max} and CE indicating a key player role probably due to its electroactive function. A co-occurrence analysis identified *Anaerovorax* sp. as a fermentative genus positively correlated to CD_{max} (0.68) and ORE (0.68). Altogether, these results provide for the first time a detailed analysis of the microbial communities resulting from different inoculation strategies and allowing to consider the reasons why the I inoculum was the most suitable for the treatment of industrial wastewater in MFC.

Ouverture

Cette étude a examiné l'impact de l'inoculation initiale sur la performance du traitement des eaux usées industrielles par les réacteurs MFC dans le but de développer le consortium microbien le plus efficace pour traiter les eaux usées industrielles. La meilleure stratégie d'inoculation était celle à partir des boues d'eaux usées industrielles (I) comparée aux autres conditions. Pour comprendre cette amélioration de performance, une analyse des communautés bactériennes a été effectuée. La structure de la communauté planctonique de la condition I était différente de celle du témoin négatif. Il semble que les consortia issus de cet inoculum étaient en concurrence avec ceux présents dans les eaux usées pour l'accès au substrat. *Chlorobium* sp. et *Sulfurovum* sp. étaient positivement corrélés avec les indices de performance (rendement Faradique pour les deux bactéries et CD_{max} pour *Chlorobium* sp.), ce qui suggère que ces deux taxa ont contribué de manière significative à l'amélioration des performances.

En ce qui concerne les biofilms anodiques, *Geobacter* sp. était prédominante dans les conditions les plus efficaces (I) et positivement corrélée au CD_{max} et le rendement Faradique indiquant un rôle clé, probablement en raison de sa fonction électroactive. Une analyse de cooccurrence a identifié *Anaerovorax* sp. positivement corrélée avec les indices CD_{max} ($r=0,68$) et ORE (DCO éliminée) ($r=0,68$). Dans l'ensemble, ces résultats fournissent pour la première fois une analyse détaillée des communautés microbiennes résultant de différentes stratégies d'inoculation et permettent de considérer les raisons pour lesquelles l'inoculum I était le plus approprié pour le traitement des eaux usées industrielles dans les MFC.

Enfin, il peut être observé que les consortia anodiques formés en milieux réels ont une diversité supérieure à ceux formés en milieux synthétiques avec des indices de Shannon multipliés par deux (de ~ 2 à ~ 4). Cela montre une haute différence de complexité. Autre différence

importante, le genre *Geobacter* était négativement corrélé aux CD_{max} dans l'étude du chapitre 4 (milieu synthétique) alors que ce même genre était positivement corrélé au CD_{max} avec des eaux usées réelles. Ces phénomènes écologiques doivent être caractérisés dans l'objectif d'une application des MFCs en conditions réelles.

6 Chapter 6. Conclusions & perspectives

The results reported in this thesis allow a better understanding of the electroactive consortia. Indeed, for the first time, a specific distribution of species of the *Geobacter* genus was observed with mainly *G. sulfurreducens* concerning acetate and lactate and a mixture between *G. toluenoxydans*, *G. pelophilus* and *G. metallireducens* in presence of propionate or butyrate. This first observation revealed the presence of a 'barrier effect' caused by *G. sulfurreducens* which rapidly colonized the anode by a competitive process but was unable to oxidize propionate, making this molecule recalcitrant over time.

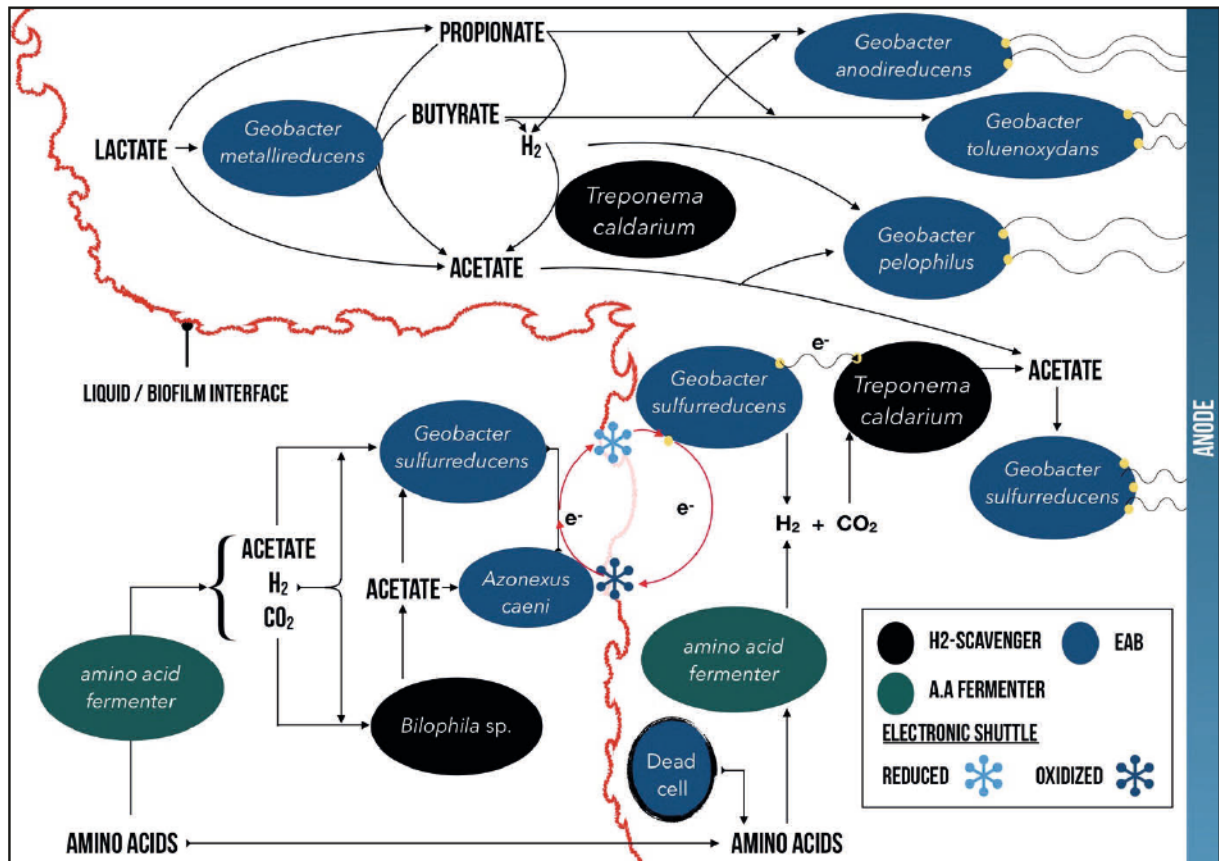
The anodic and planktonic consortia were analyzed from the core-microbiome approach, with the objective of establishing metabolic networks of bacteria common to electroactive biofilms and common to the bulks. This analysis revealed two consortia, one planktonic composed of eight bacteria and the other anodic composed of four bacteria. One of the core biofilm OTUs was identified as a *Treponema caldarium* whose abundance was strongly negatively correlated with current density confirming that this genus could be used as a bioindicator of a loss of biofilm electroactivity. An in-depth ecological analysis showed a probable implication in the establishment of an homoacetogenic pathway in electroactive biofilms, less effective for the conversion of short-chain acids than direct anode oxidation by EABs. This study also shows that amino acid fermenters were still present with *G. sulfurreducens* in the anodic and planktonic habitat. In planktonic environment, *G. sulfurreducens* and amino acids fermenters i.e., *Lutispora thermophila*, *Paraclostridium*

benzoelyticum, *Macellibacteroides fermentans*, had very significant correlations ($r=0.79$ to $r=0.93$).

Pre-acclimation techniques for anodic consortia have shown a significant improvement in CE. Correlation analyses showed that this improvement was due to an increase in the abundance of *Treponema caldarium*, a subdominant bacterium, and the decrease in *Geobacter anodireducens*. The link of the different studies shows that *T. caldarium* was involved in an homoacetogenic pathway into electroactive biofilms, which decreased the CD_{max} but, at the same time, would allow a better use of H_2 which improves CE. So, when a bacterium with a high metabolic versatility is present, such as *G. anodireducens*, it colonizes the anode whatever the substrate. Therefore, the use of the substrate to pre-acclimate bio-anodes may be unsuccessful to improve the propionate oxidation rate.

Work carried out in MFCs according to different inocula and industrial wastewaters in substrate showed a significant relationship between the presence of *Geobacter* sp. on bio-anodes and the CD_{max} . This advance is important to consider the ecological engineering techniques to improve the performances of MFCs with real wastewaters. This study allows to compare the difference in diversity between synthetic and real consortia with a doubling of the Shannon index (from ~ 2 to ~ 4). A first common point revealed the presence of *Geobacter* sp. as main electroactive bacteria. However, the rest of the diversity was not very similar due to complex wastewaters.

Taken together, results of this thesis make it possible to establish the metabolic network xxx :



Schematic 6-1 Hypothetical metabolic network showing the different bacterial taxa identified during this thesis and their role at the planktonic and anode levels.

In the microbiological analysis in Chapter 3, electronic shuttles have been considered as electron vectors to the anode. However, *G. sulfurreducens* could also use fermentative bacteria as terminal electron acceptor. This would explain the co-occurrence pattern and the specific links of planktonic core-microbiome. It seems that a precise selection between an electroactive bacterium and a fermentative is favored by the electronic interaction. As *G. sulfurreducens* can transfer its electrons to any conductive elements (anode, e-pili, cytochromes) it is therefore the ability of the fermentative bacteria to uptake the electrons that would determine the specificity of the interaction types (Fig. 6-1).

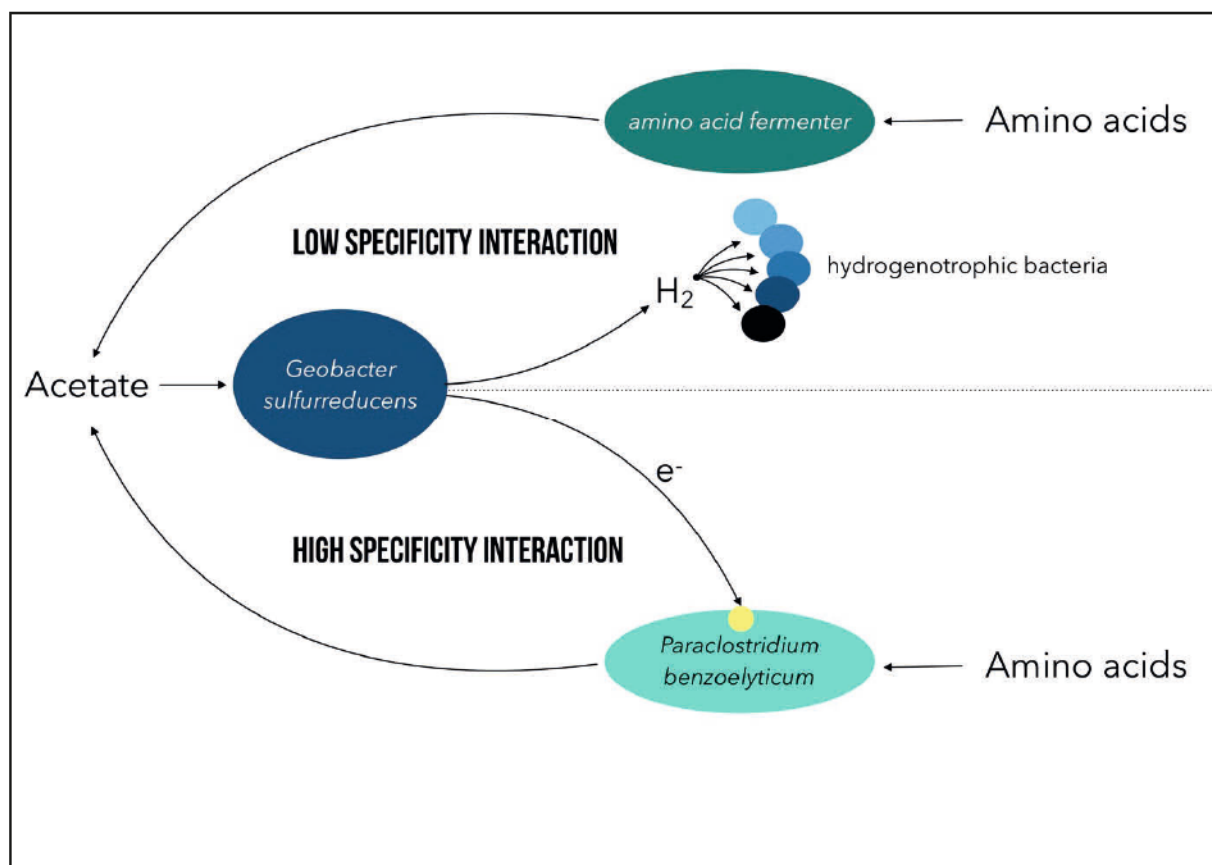


Fig. 6-1 Hypothetical network of interaction between amino acid fermenters either by hydrogen electron transfer or by direct electron transfer.

In order to better understand the link between amino acid fermenters and electroactive bacteria, the following experiments could be considered: Implement amino acid fermentation with and without *G. sulfurreducens*. Then, determine for each condition the core-microbiomes and compare their differences. If these are different this would indicate a specific interaction. In this case, co-culture could be tested with *G. sulfurreducens* and the amino acid fermenters identified to determine precisely the type of electron transfer, i.e. hydrogen or direct electron transfer.

Complementarily, a co-culture between *T. caldarium* and *G. sulfurreducens* would allow to characterize precisely the relation of these two species, found together on the anode core-microbiome of MECs fed with short-chain acids. A microscopic analysis would allow to know if the homoacetogenic pathway is set up during the syntrophy with *Geobacter sulfurreducens* which is not directly connected to the anode. It would be interesting to determine if the electron transfer between these two bacteria is direct (pili or cytochrome type) or mediated (via hydrogen).

The experiment presented in chapter 4 presented anodic pre-acclimation based on defined substrates. This ecological strategy has not been effective in improving the oxidation rate of propionate due to the presence of an electroactive bacterium with high metabolic versatility. In order to avoid this problem, it could be considered anodic pre-acclimation with either pure strains or synthetic ecosystems that would offer both high current densities by minimizing the installation of an homoacetogenic pathway and high CE with an efficient use of hydrogen. A co-culture between *G. anodireducens* and *Hydrogenophaga electricum* seems to be a promising prospect, with a synthetic consortium capable of oxidizing many organic acids and a direct oxidation of H₂ at the anode without the establishment of an homoacetogenic pathway.

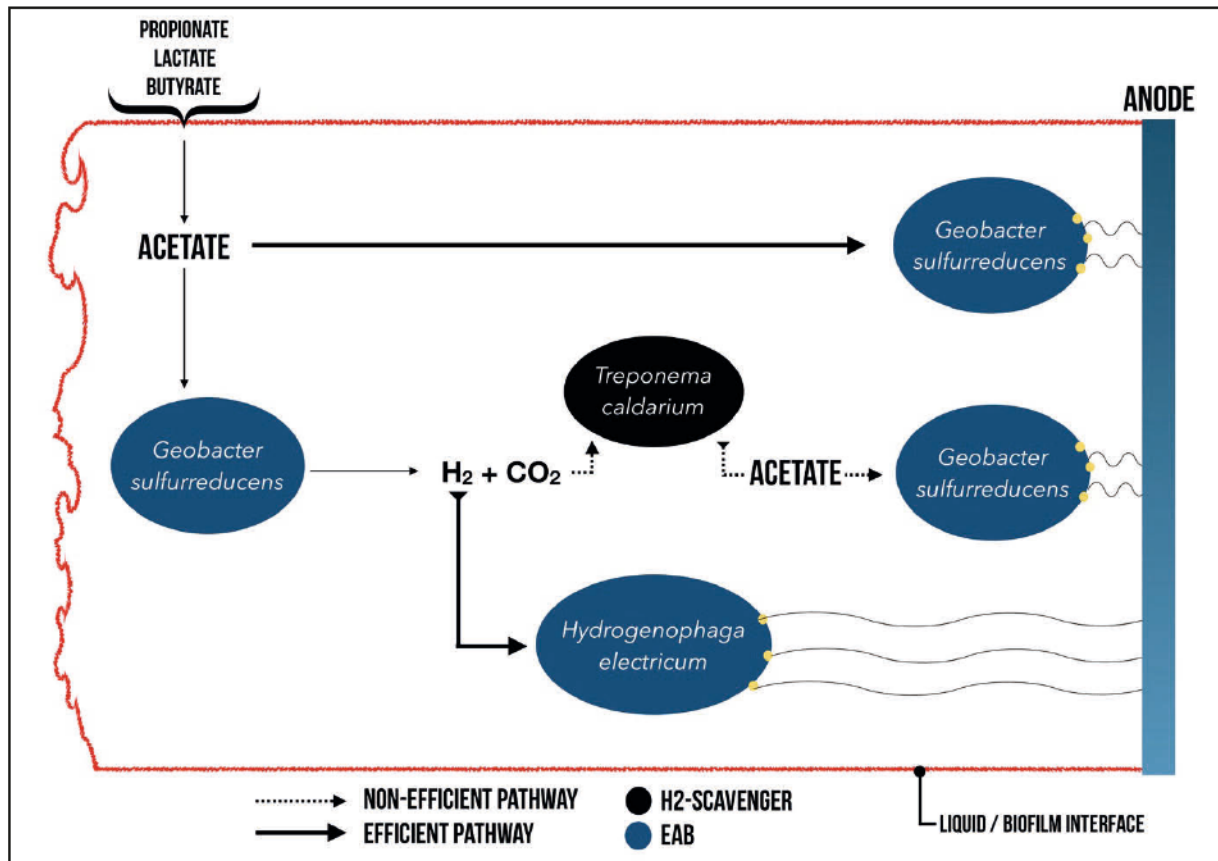


Fig. 6-2 Putative interaction network showing the beneficial effect of an hydrogenotrophic electroactive bacteria compared to a homoacetogen in an electroactive biofilm.

It is evident that the ecological fundamentals revealed by studies in synthetic media constitute an important first step to understand the behavior of the consortia in real conditions. The experience of Chapter 5 has shown the great difference in diversity between synthetic and complex media. Currently, it seems necessary to carry on the studies in a synthetic medium and gradually increasing its complexity (e.g. by increasing substrate diversity) to have a detailed view of ecological interactions within electroactive consortia. Next, these ecological fundamentals will serve as a tool for understanding consortia fed with complex wastewater,

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with the aim of proposing effective ecological engineering strategies and improving MXCs for wider application.

7 Appendix

In this section, the supplementary information from the previous chapters are presented.

7.1 Chapter.2 Microbial anodic consortia fed with fermentable substrates in microbial electrolysis cells: significance of microbial structures

Table S 1 OTUs classification according to BLASTn (NCBI) results

OTU n°	Taxa	Identity (%)
1	<i>Geobacter sulfurreducens</i>	100
5	<i>Geobacter metallireducens</i> GS-15	99
8	<i>Treponema caldarium</i>	99
9	<i>Acetobacterium hydrogenigenes</i>	86
12	<i>Geobacter pelophilus</i>	97
15	<i>Seleniivibrio woodruffi</i>	95

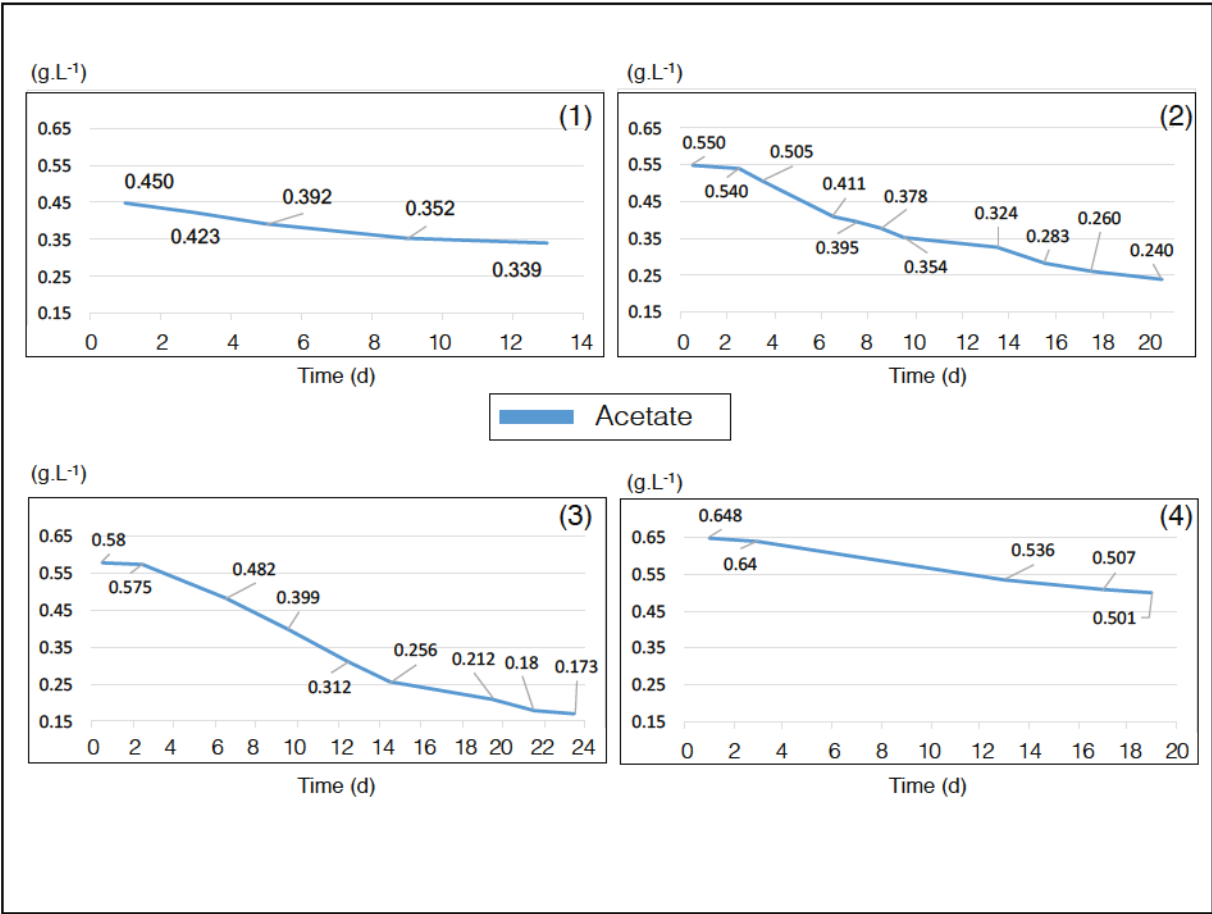


Fig.S 1 Metabolite concentrations (g/L) during acetate-fed MECs assays over time (d) according to the replicate numbers (1-4)

Appendix

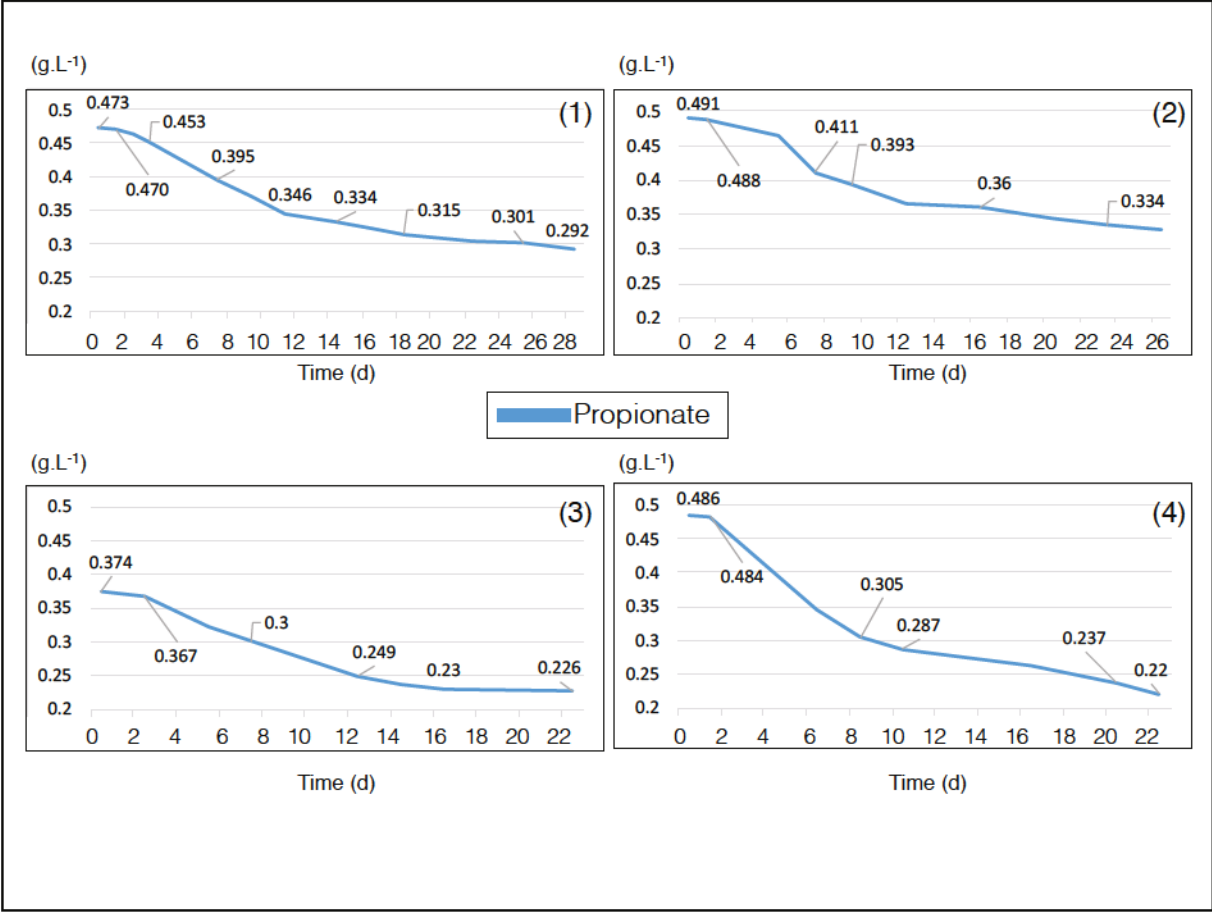


Fig.S 2 Metabolite concentrations (g/L) during propionate-fed MECs assays over time (d) according to the replicate numbers (1-4)

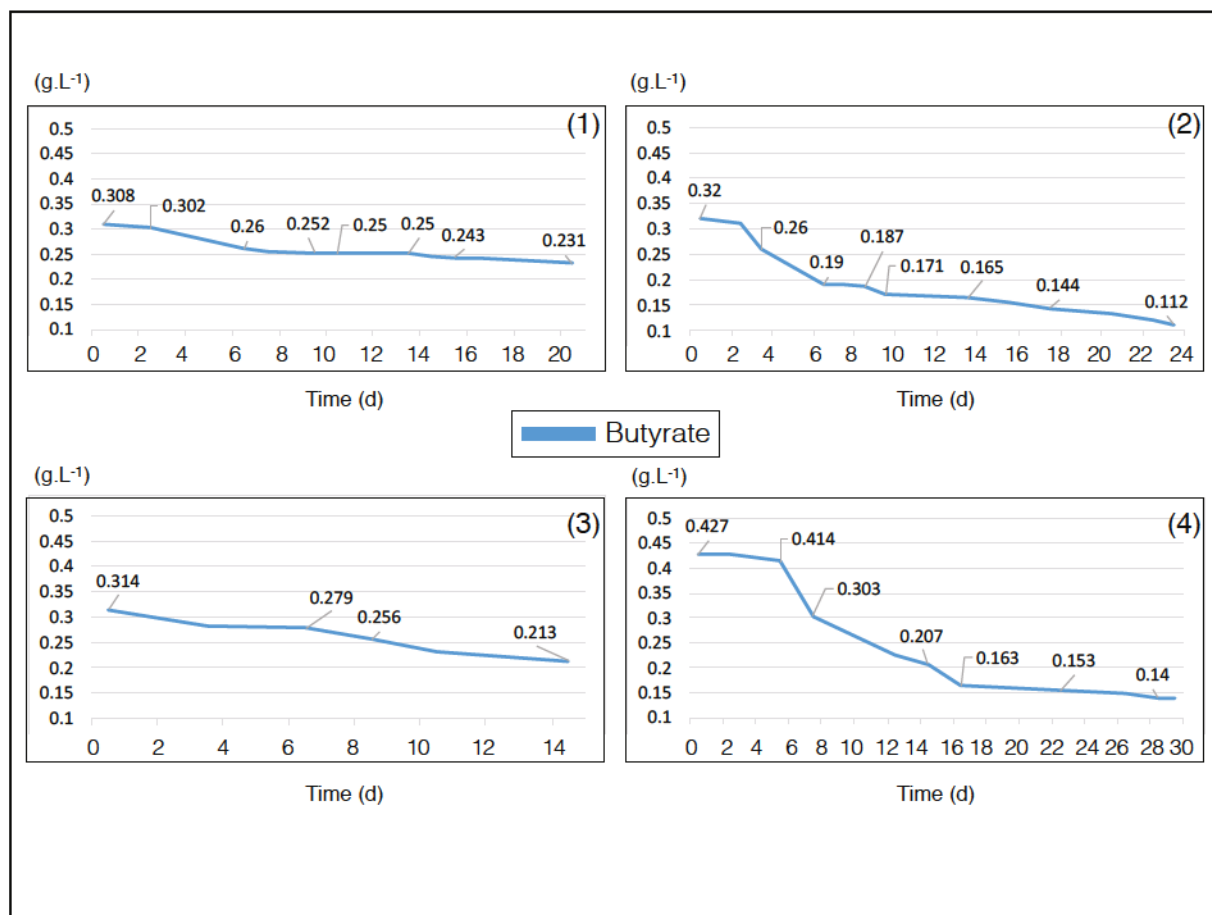


Fig.S 3 Metabolite concentrations (g/L) during butyrate-fed MECs assays over time (d) according to the replicate numbers (1-4)

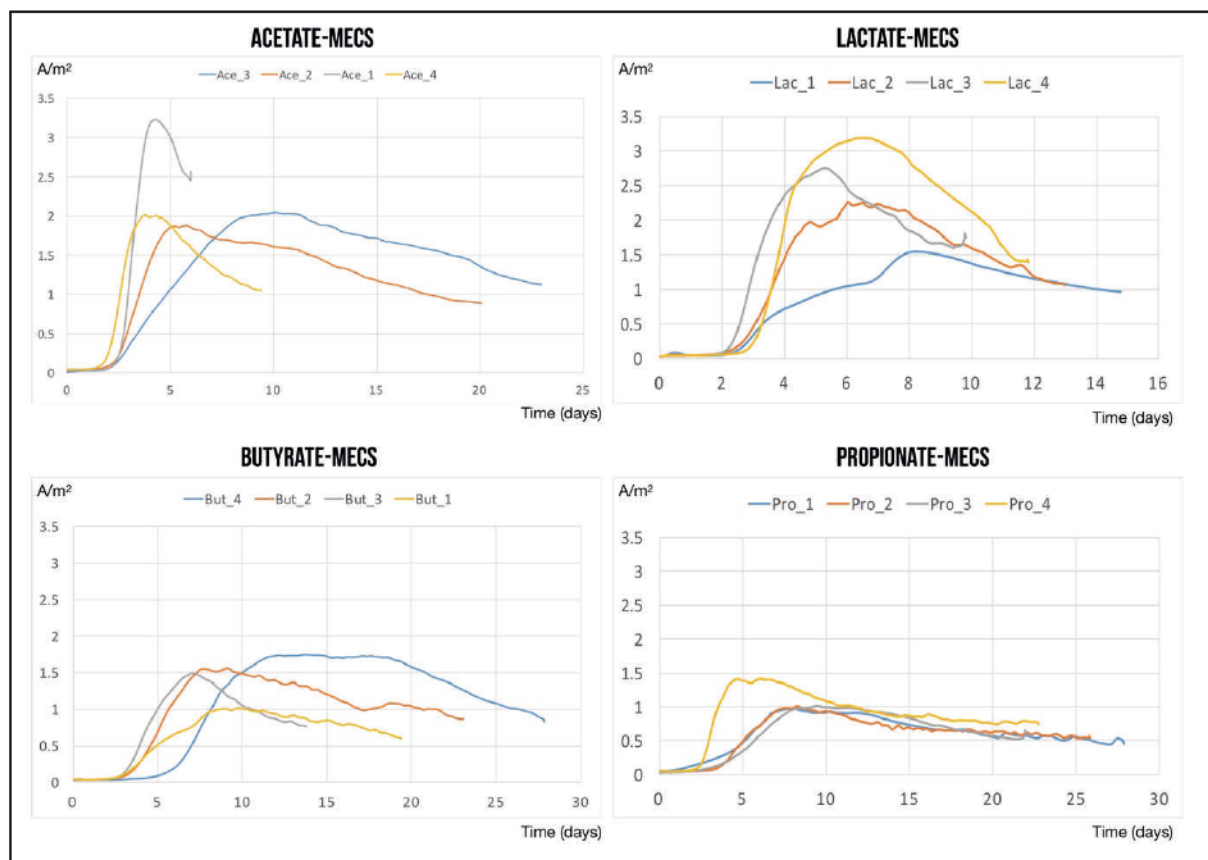


Fig.S 4 Current density curves for each MEC.

7.2 Chapter.3 A core-microbiome approach to identify key microbes and interactions in electroactive microbial communities

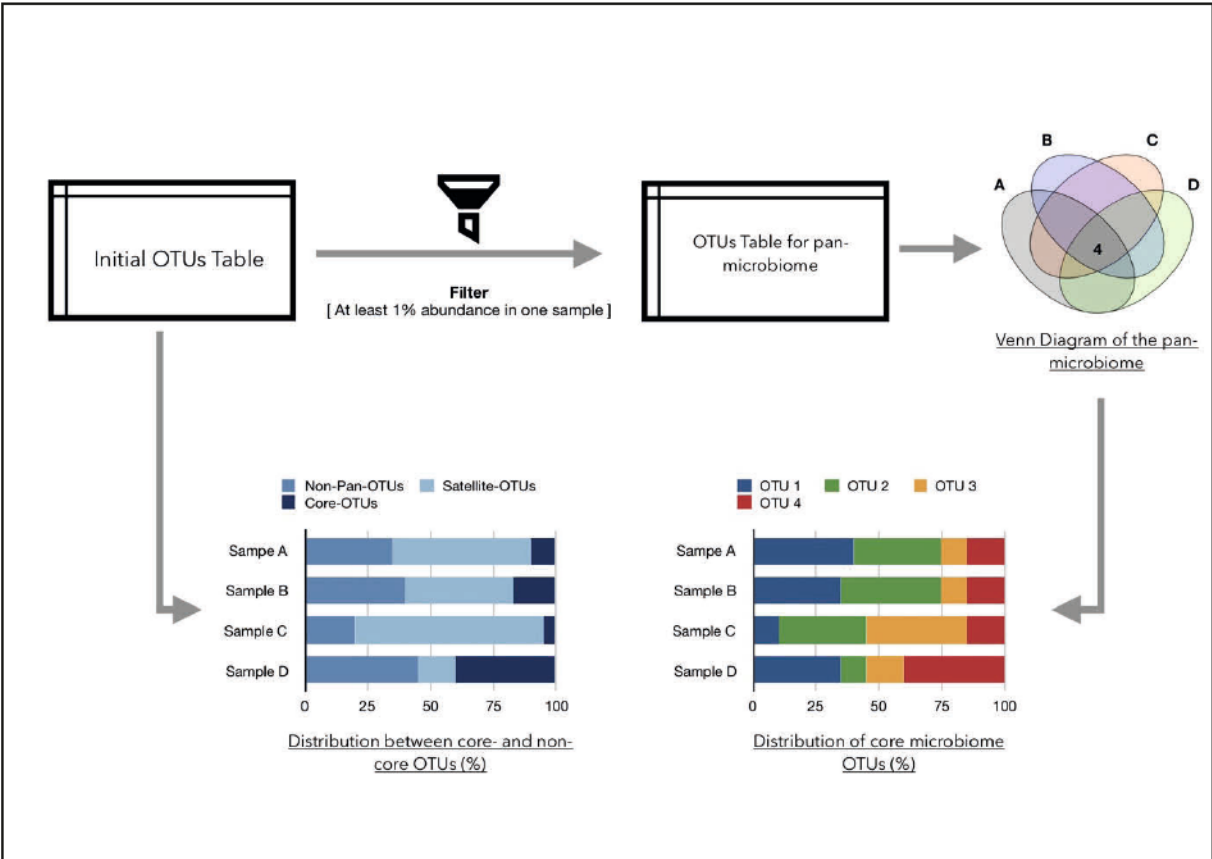


Fig.S 1 Details of filtering procedure from initial OTUs dataset to Venn diagram

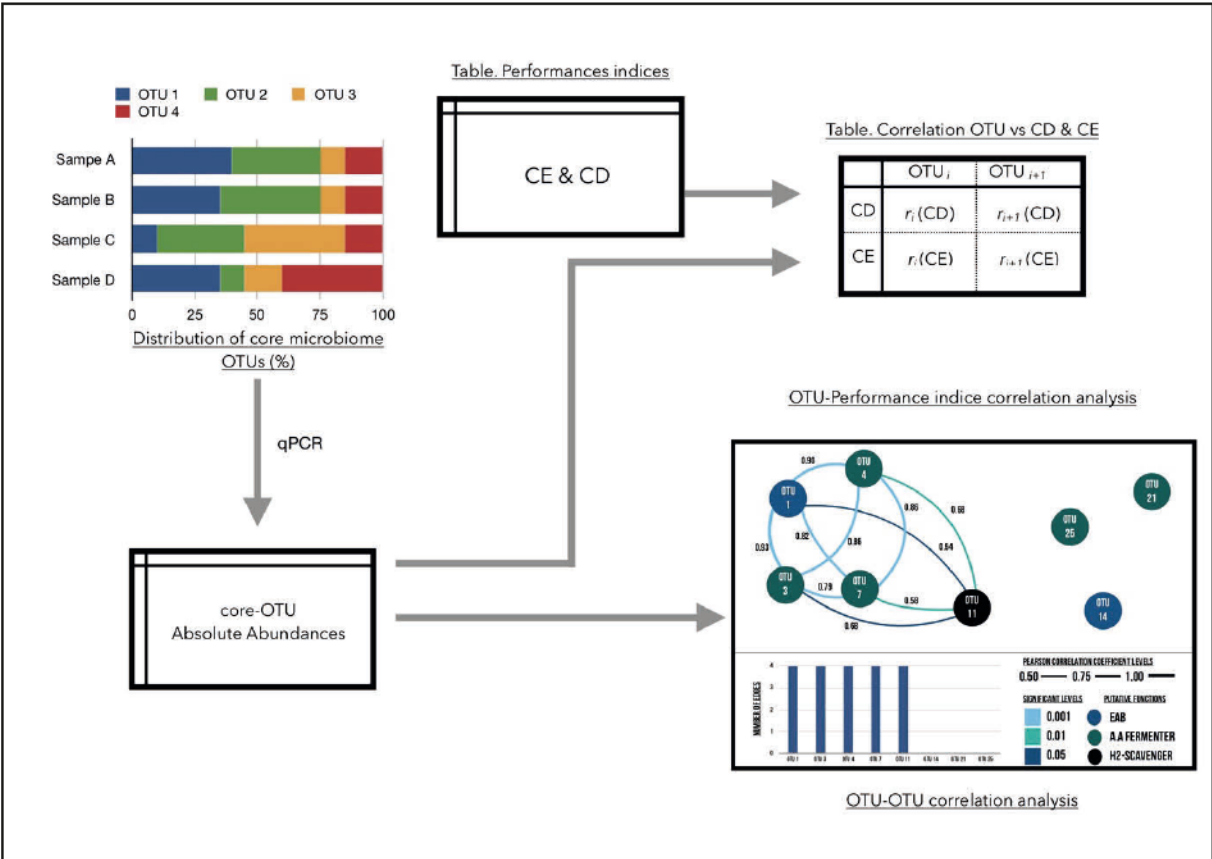


Fig.S 2 Detail of the correlation analysis procedure either between OTUs or between OTUs and performance indices (CE: Coulombic efficiency; CD: Current density). Data are normalized before correlations.

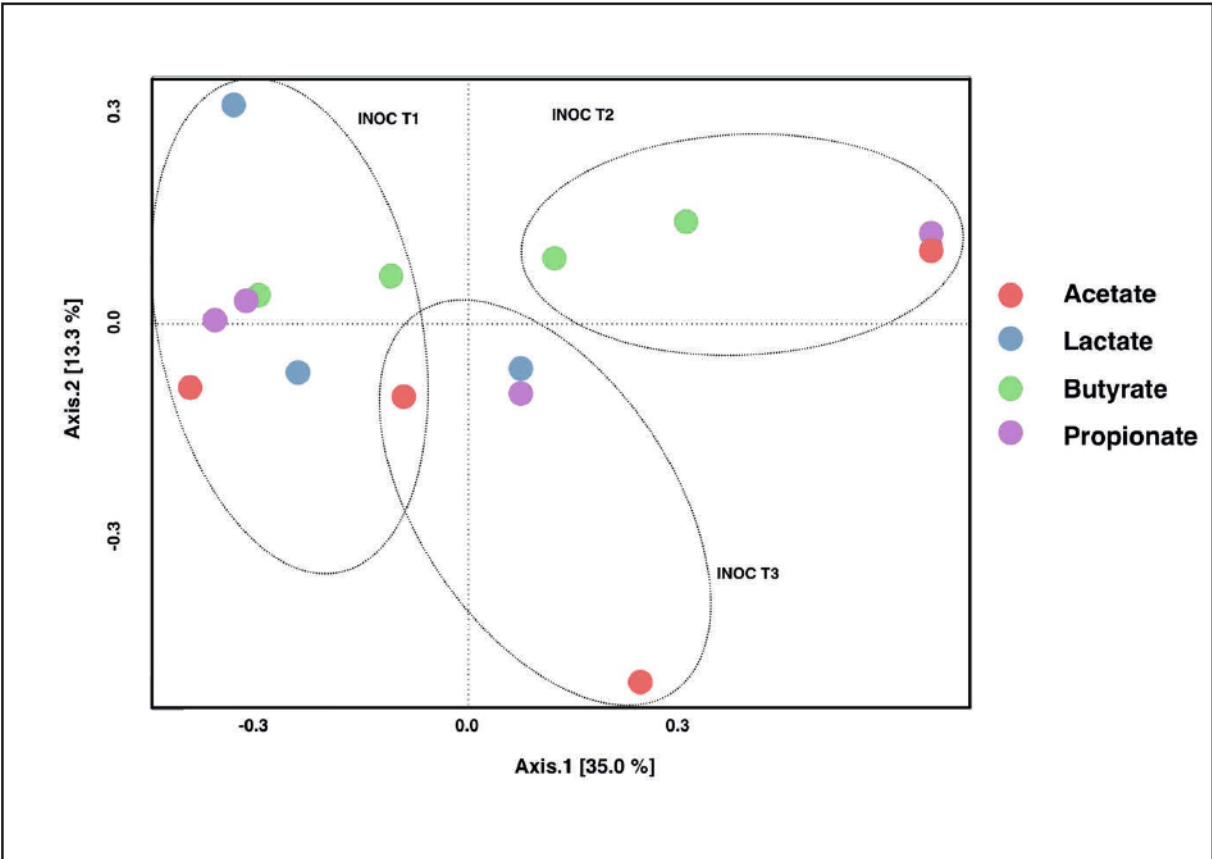


Fig.S 3 Principal coordinate analysis (PCoA) based on weighted-UniFrac distance matrix showing the overall distribution pattern between the inocula samples according to the sampling times at source (T1, T2 and T3). One sample correspond to the initial point of one batch. The pattern of separation by clusters have a significant difference calculated by the permutation test ($n = 9999$, P .value = 0.001)

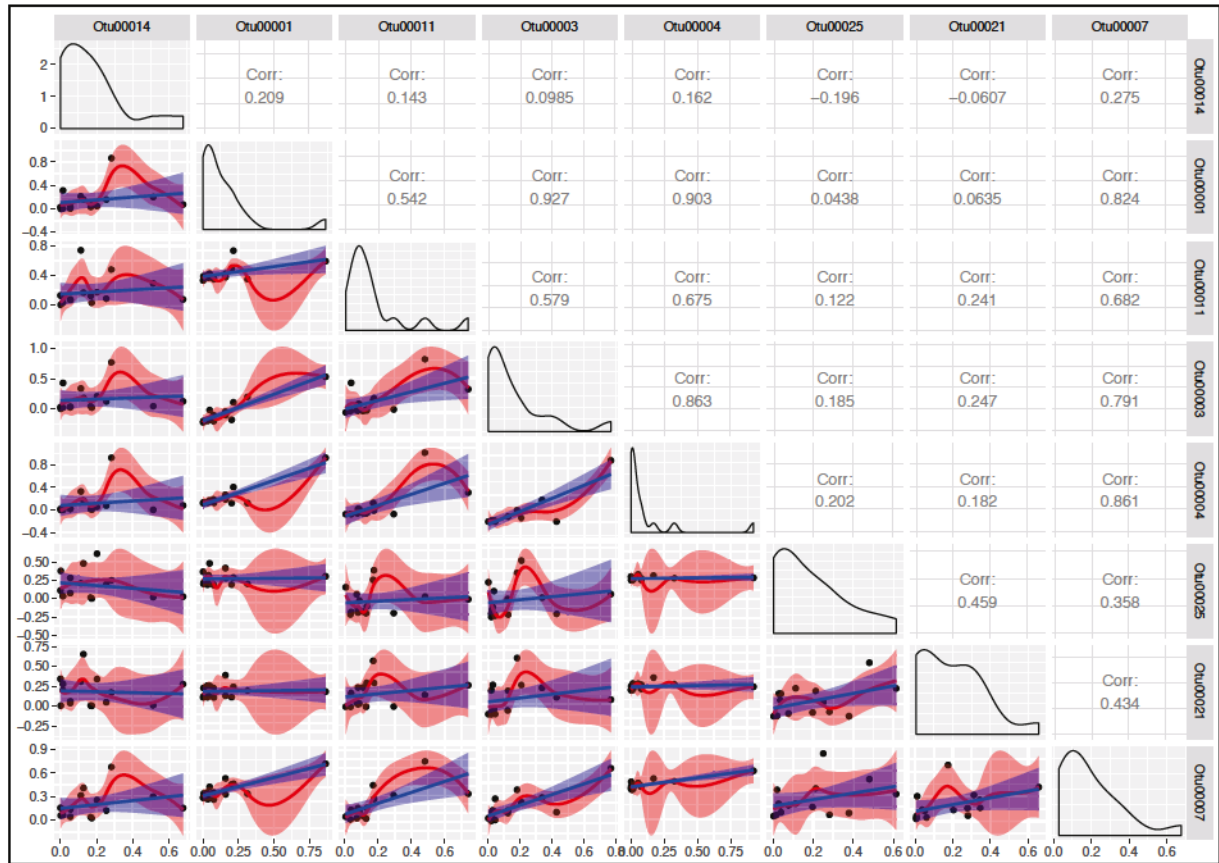


Fig.S 4 Correlation diagram between absolute abundances of planktonic core-OTUs normalized. On the right top, absolute value of the correlation (Pearson coefficient) plus the result of the correlation test. On the bottom left, the bivariate scatterplots with a fitted line and on the diagonal line histograms of the OTU frequency as a function of normalized abundances.

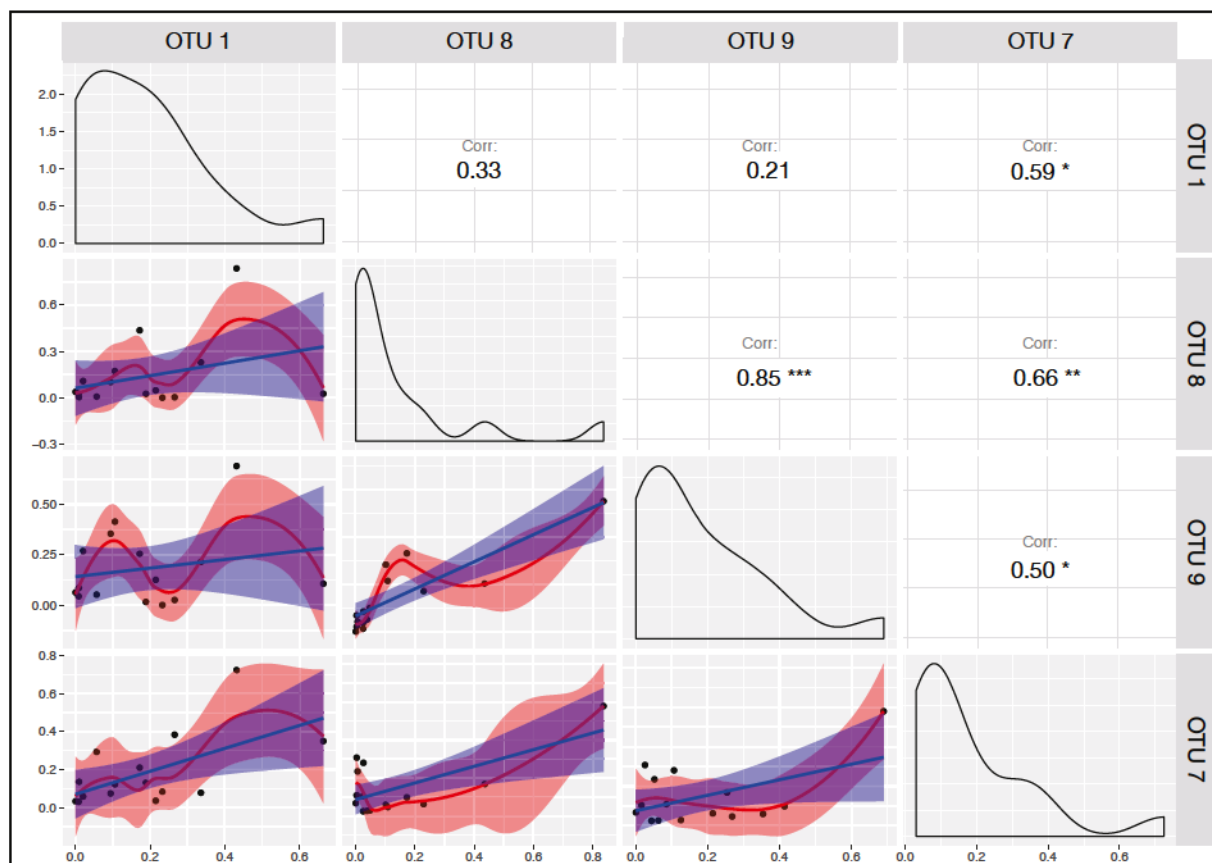


Fig.S 5 Correlation diagram between the absolute abundances of the biofilm core-OTUs normalized. On the right top, absolute value of the correlation (Pearson coefficient) plus the result of the correlation test. The more asterisks, the more significant relation

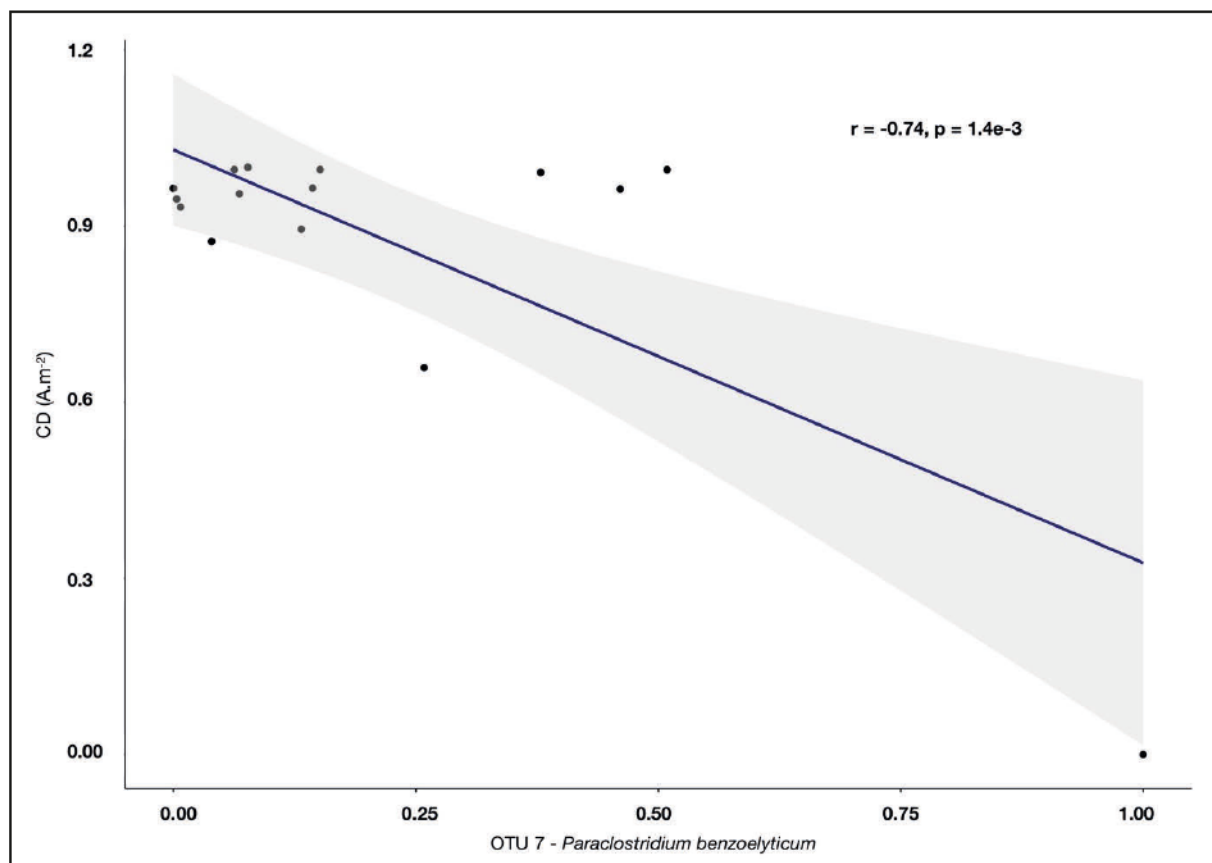


Fig.S 6 Correlation between the normalized absolute abundance of OTU 7 close to 100% of *Paraclostridium benzoelyticum* and the normalized current density production (A.m⁻²) of

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microbial electrolytic cells. The correlation line is shown in blue and the 95% confidence interval in grey.

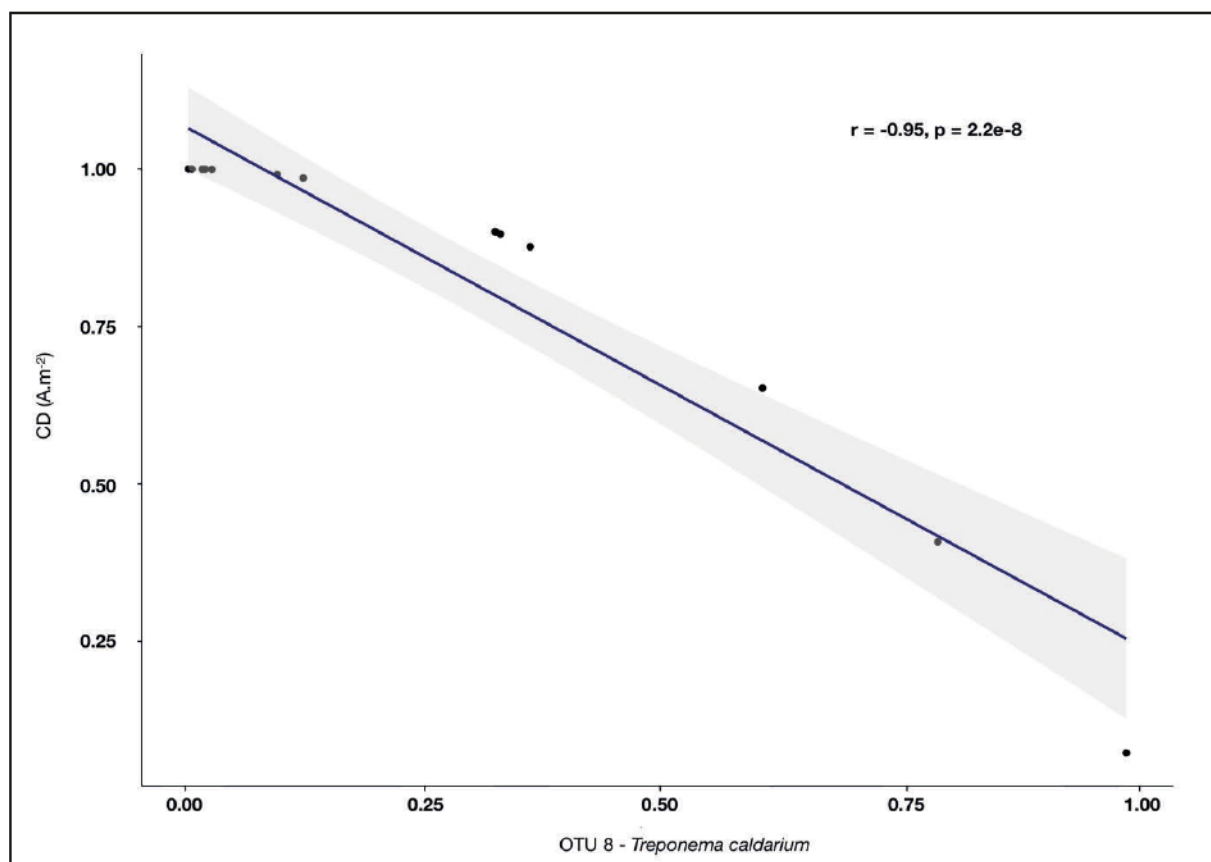


Fig.S 7 Correlation between the normalized absolute abundance of OTU 8 close to 99% of *Treponema caldarium* and the normalized current density production (A.m⁻²) of microbial electrolytic cells. The correlation line is shown in blue and the 95% confidence interval in grey.

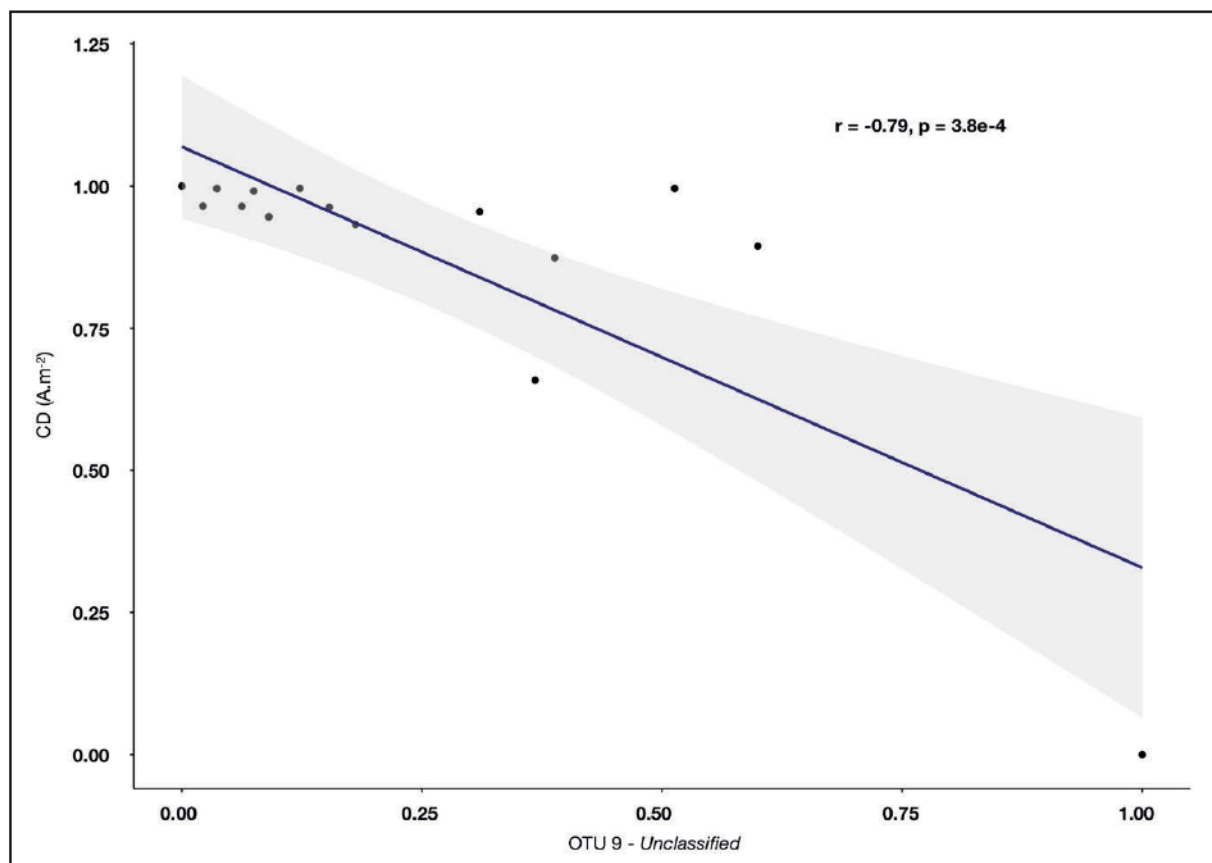


Fig.S 8 Correlation between the normalized absolute abundance of OTU 9 (*Unclassified*) and the normalized current density production (A.m⁻²) of microbial electrolytic cells. The correlation line is shown in blue and the 95% confidence interval in grey.

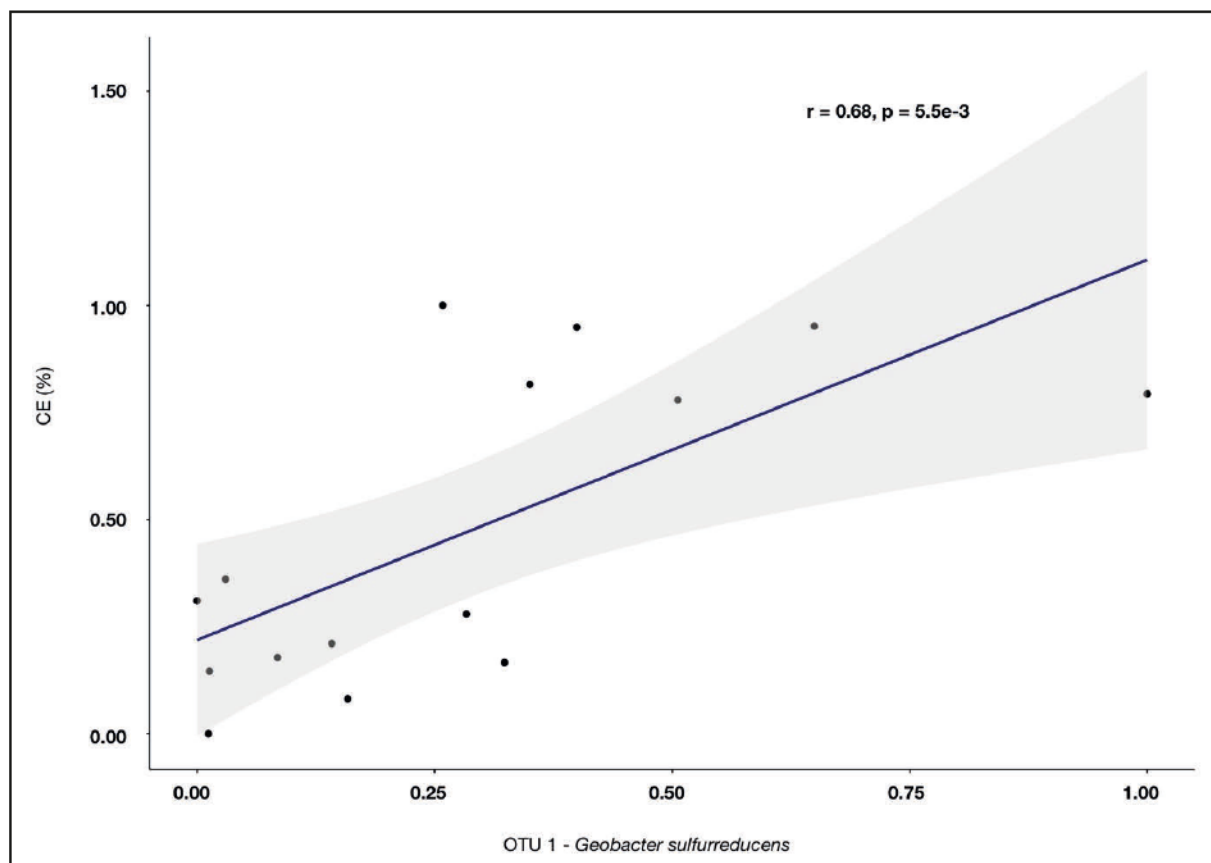


Fig.S 9 Correlation between the normalized absolute abundance of OTU 1 close to 100% of *Geobacter sulfurreducens* and the normalized coulombic efficiency (%) of microbial electrolytic cells. The correlation line is shown in blue and the 95% confidence interval in grey.

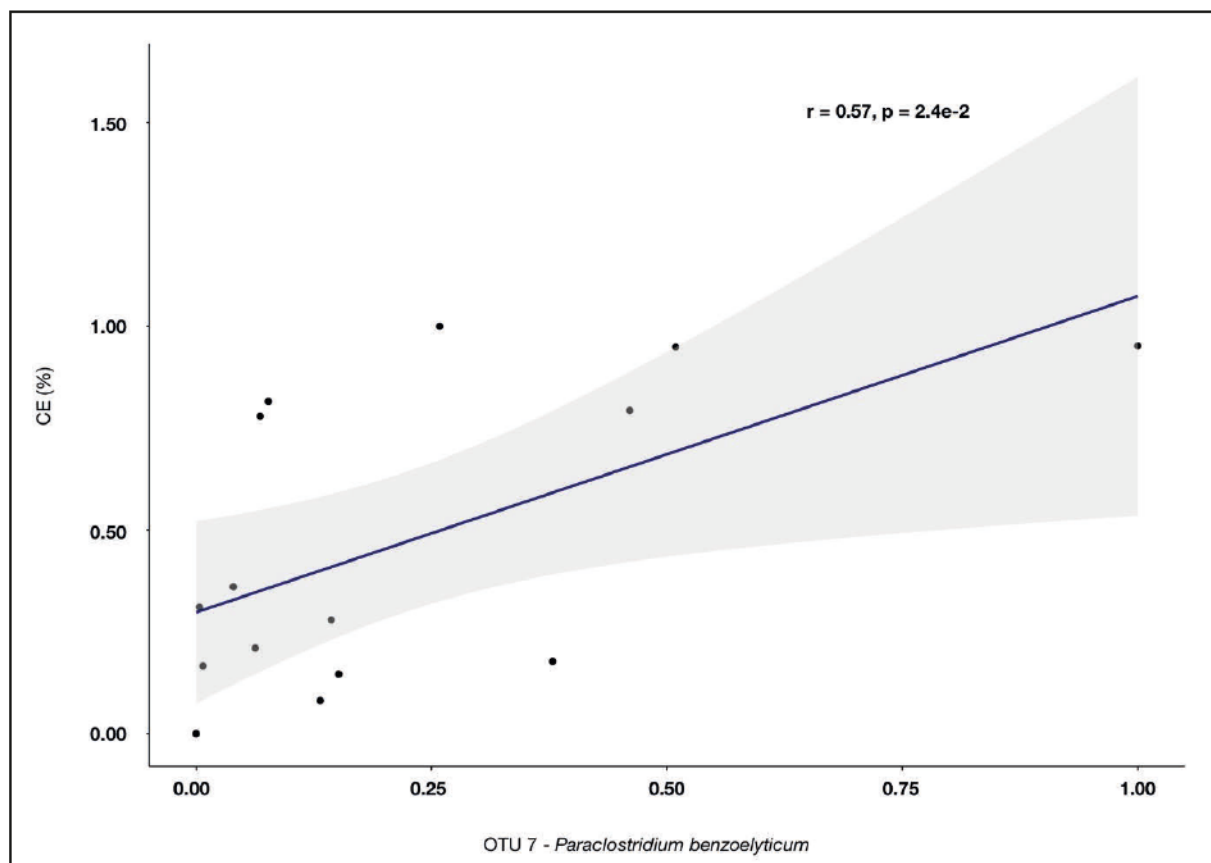


Fig.S 10 Correlation between the normalized absolute abundance of OTU 7 close to 100% of *Paraclostridium benzoelyticum* and the normalized coulombic efficiency (%) of microbial electrolytic cells. The correlation line is shown in blue and the 95% confidence interval.

7.3 Chapter.4 Anodic pre-acclimations reveals the essential role of subdominant bacteria in the performance of microbial electrolysis cells

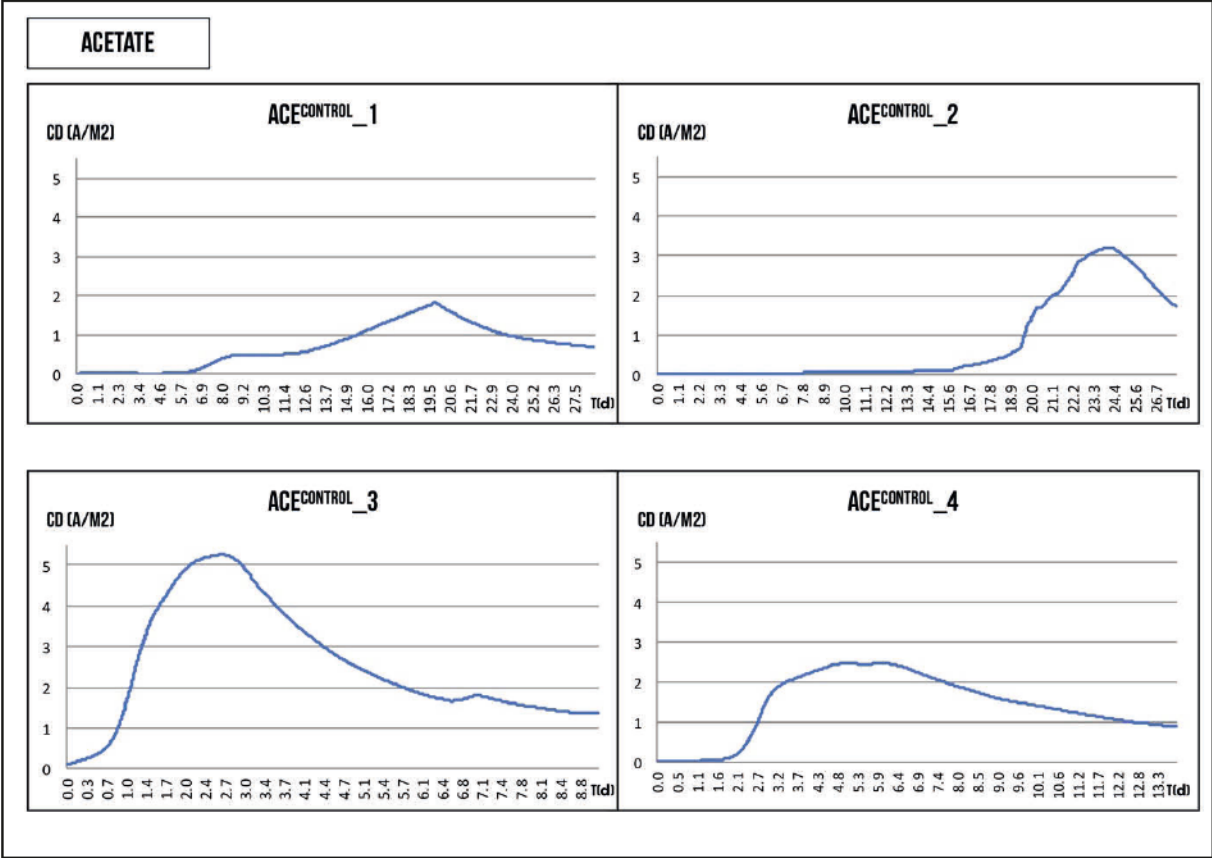


Fig.S 1 Current density curves of ACE^{control} condition.

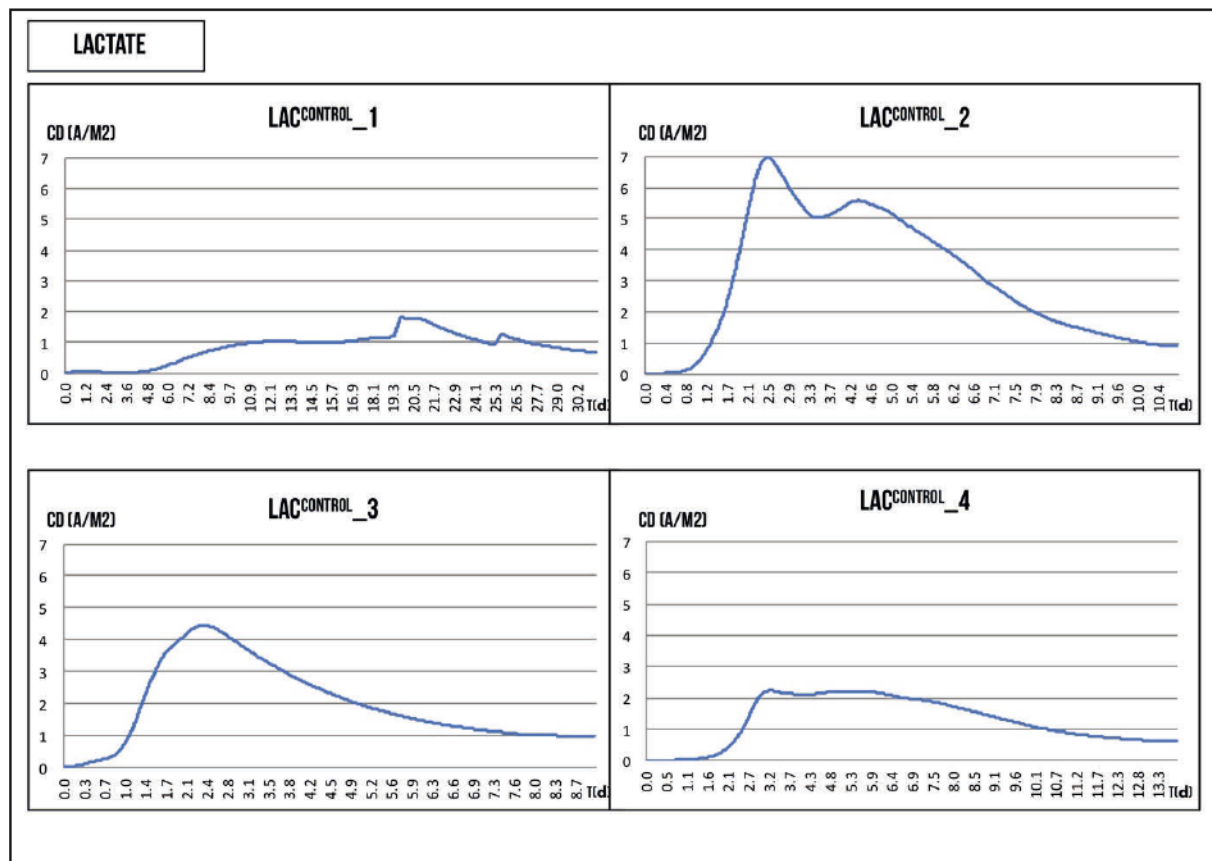


Fig.S 2 Current density curves of LAC^{control} condition.

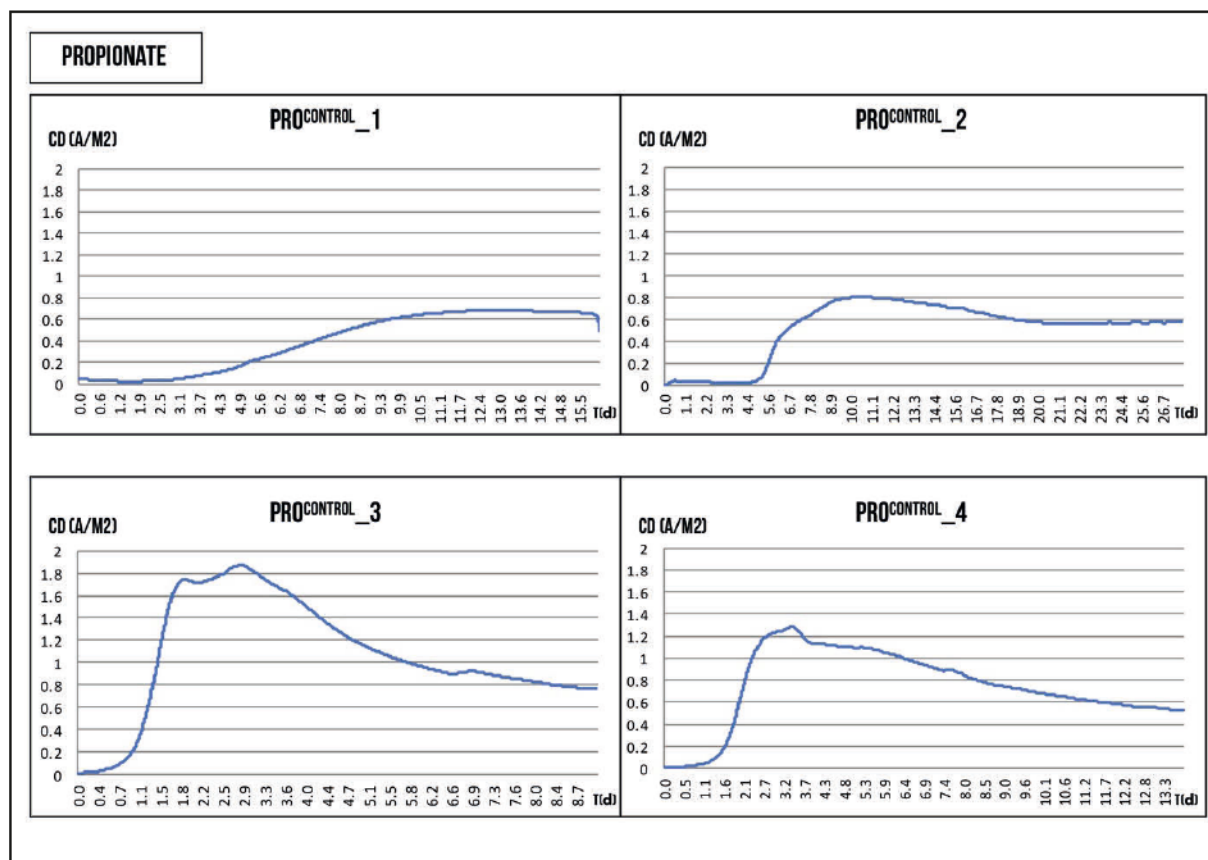


Fig.S 3 Current density curves of PRO^{control} condition.

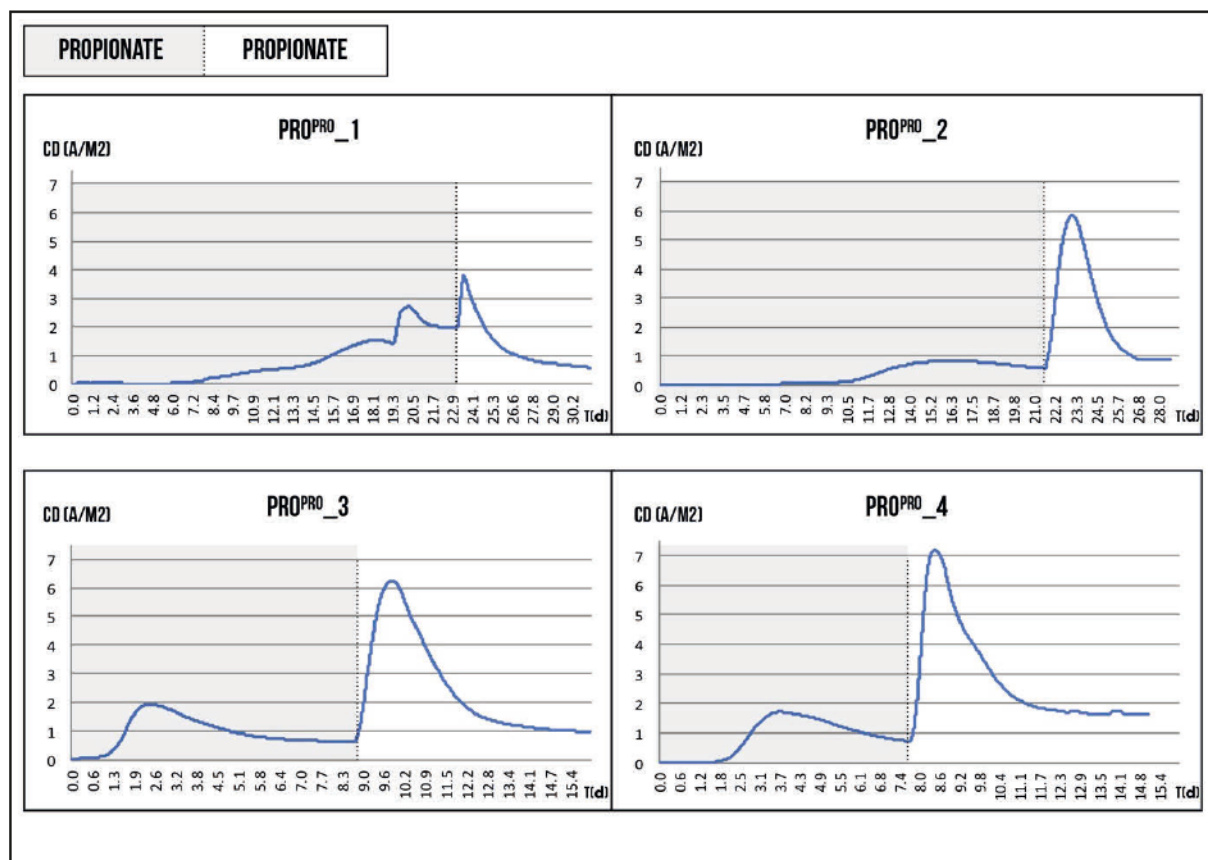


Fig.S 4 Current density curves of PRO^{PRO} condition.

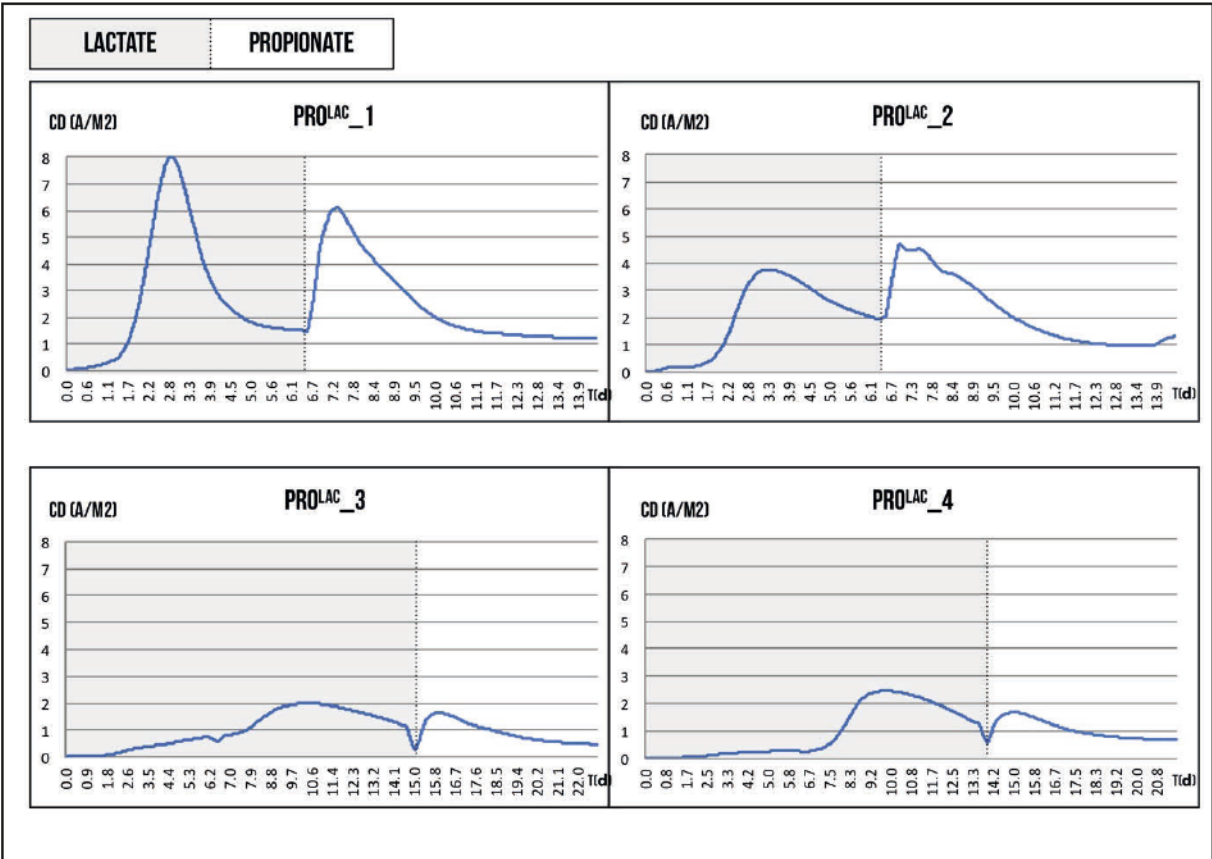


Fig.S 5 Current density curves of PRO^{LAC} condition.

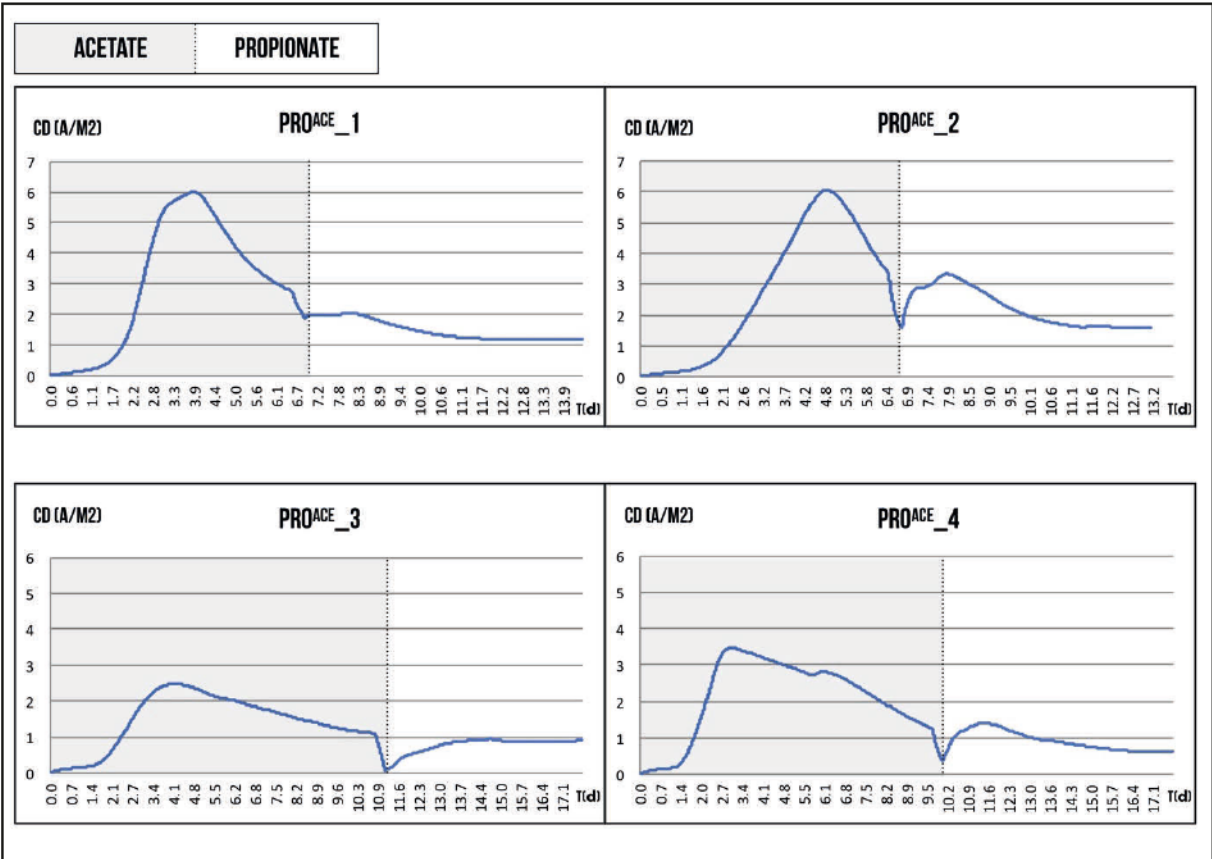


Fig.S 6 Current density curves of PRO^{ACE} condition.

7.4 Chapter.5 On the choice of a proper inoculum for treating industrial wastewater with BES

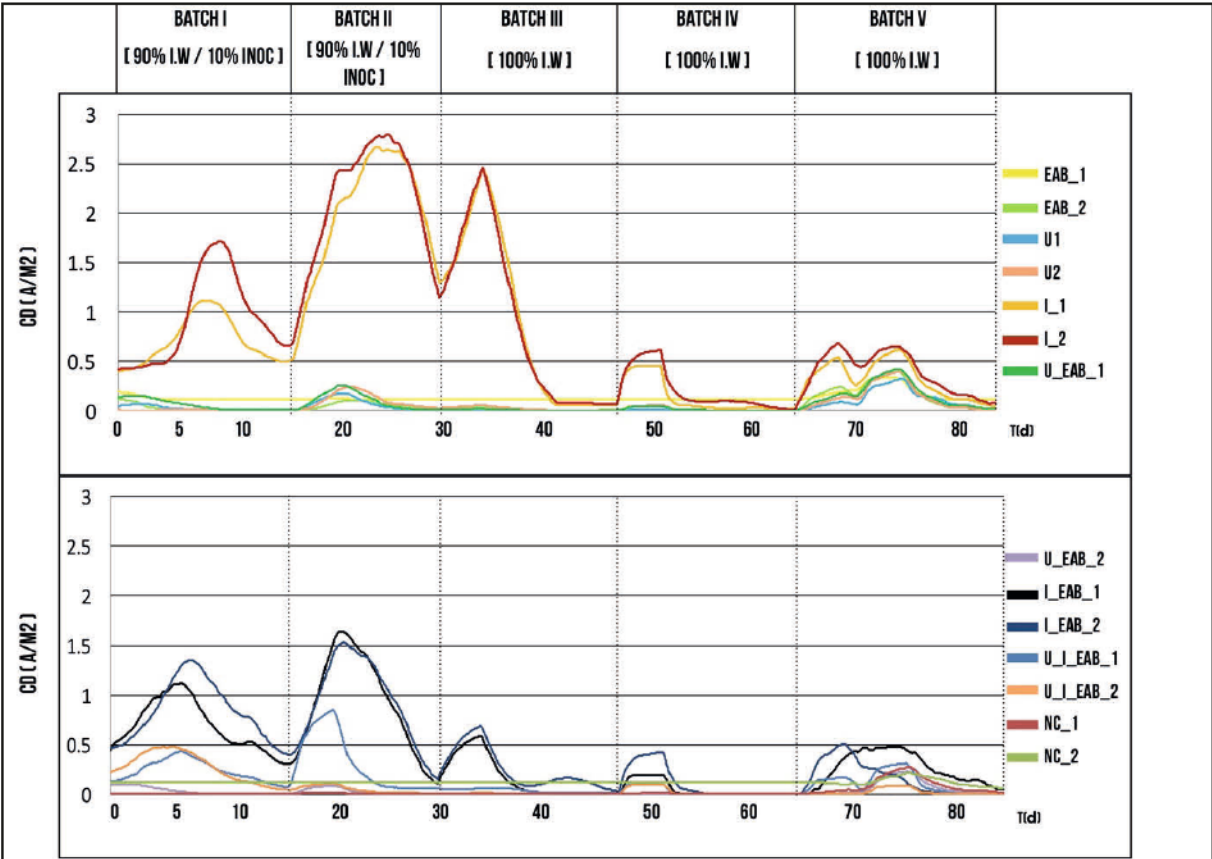


Fig.S 1 Current densities of the 5 batches.

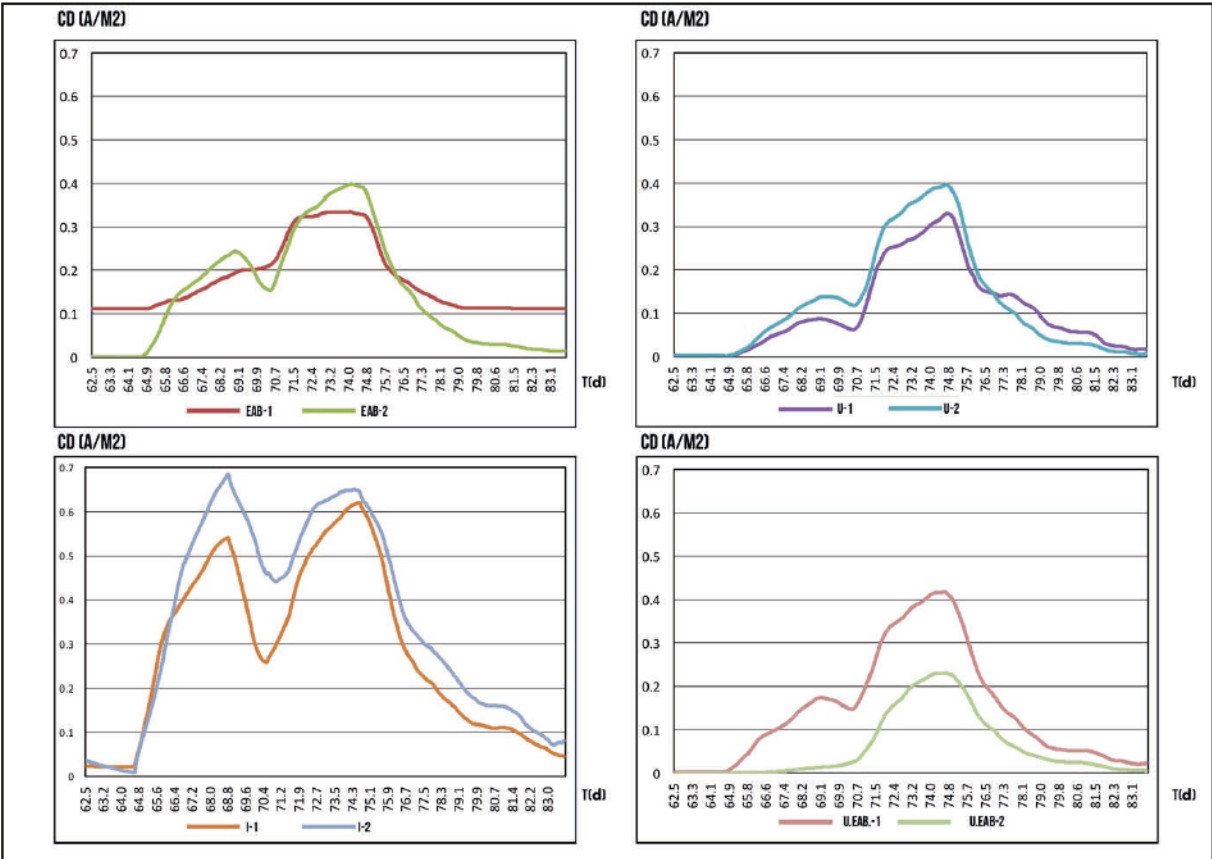


Fig.S 2 Current densities of the last batch corresponding to the conditions EAB, U, I & U_EAB.

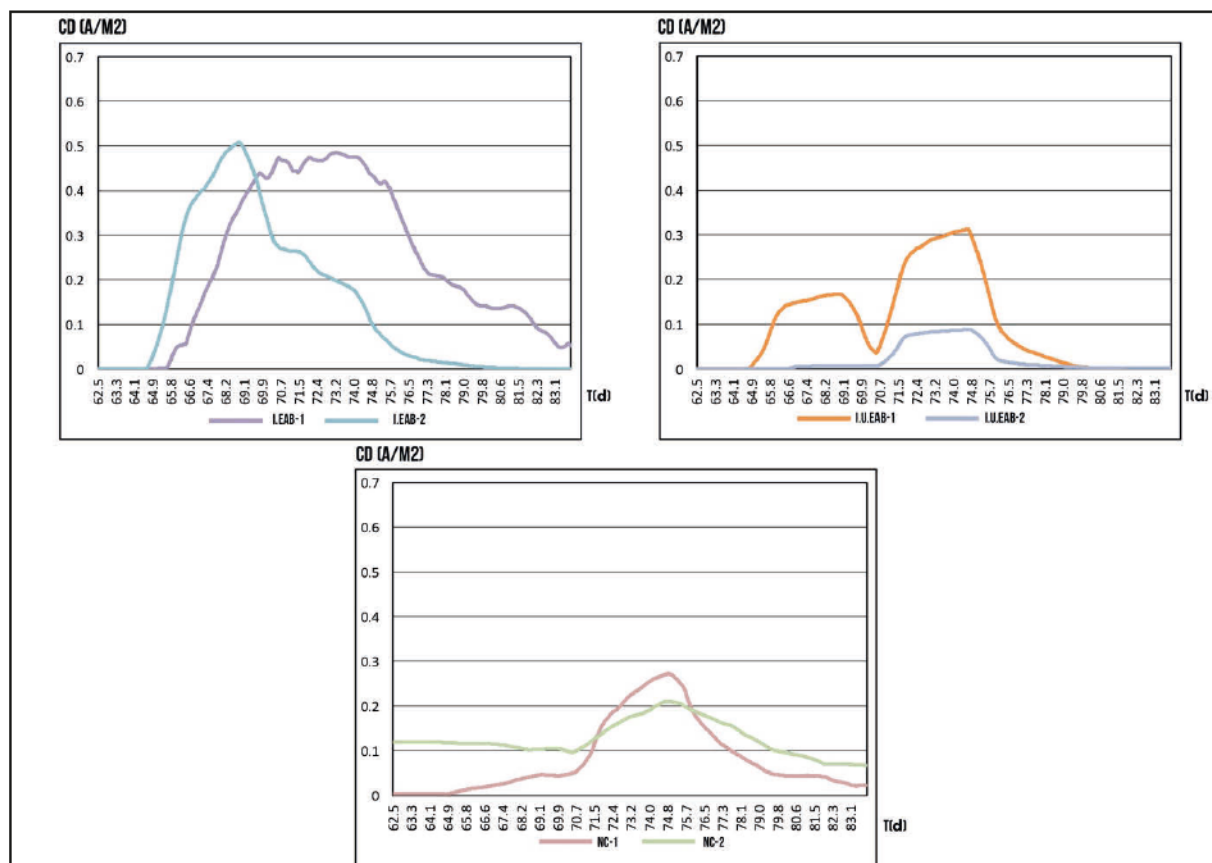


Fig.S 3 Current densities of the last batch corresponding to the conditions I_EAB, I_U_EAB & NC.

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