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

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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 mMe^- 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

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 H_2, microbial electrolysis cells (MECs) constitute a very promising solution. In MEC, the organic matter contained in wastewaters is oxidized at the anode in CO_2, 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) [1, 2]. 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 [3]. 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 [4]. These biofilms are mostly composed of EABs able to use an anode as final electron acceptor [5]. 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 [6]. 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 [7]. This implies a critical interdependence between a producer and a consumer, so, called syntrophy [8]. 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 [9]. Some EABs such as *Geobacter metallireducens,* can even

convert directly fermentable substrates into electrons without syntrophic partners [10]. 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) [11]. 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 [12, 13]. Among the already well known EABs, *Geobacter sulfurreducens* is often found dominant in ecosystems fed with either acetate or lactate as sole electron donor [14, 15]. Concerning propionate, the presence of *Geovibrio ferrireducens* was revealed by DGGE in microbial fuel cell (MFC) [16]. 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 [17].

2 Materials & Methods

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 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 N_2 gas (purity $\geq$ 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.

2.3 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 cm^2 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.

2.4 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.

2.5 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 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 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.

2.6 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 [18]. All sequences were submitted to Genbank under accession numbers MG238597 - MG241108.

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).

2.8 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].

2.9 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 [19]. 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.

3 Results & discussion

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 ± 0.22 $A.m^{-2}$ respectively) (Fig. 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. 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 [13, 20].

In terms of coulombic efficiencies (CE), (average of the 4 MECs per substrate), acetate-fed MECs showed the highest values (84.7 ± 1.43%) as widely observed in other studies (Fig. 1 – b) [16, 21]. Lactate-fed MECs had an average CE of 77.5 ± 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.

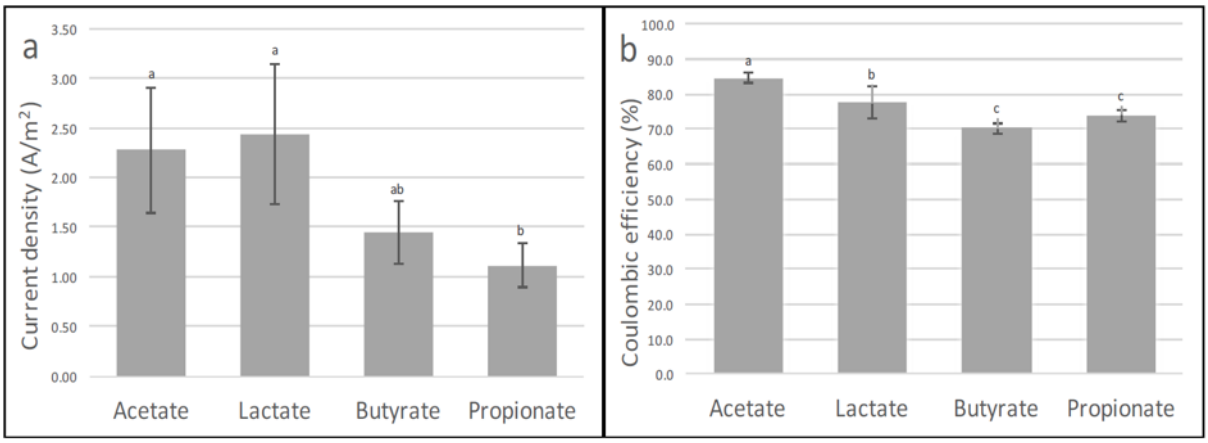


Fig. 1 Performance of MECs (average of the batch cycles of the duplicate (n=4) reactors in term of a current density of the maximum current density and b coulombic efficiency. Samples with the same letter (a, b or c) have no significant difference.

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 1). Metabolite concentrations over time are shown in Fig. 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. 1-b) and the addition of methanogenesis inhibitor (2-BES) which prevents electron leakage to methane.

Table 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

204

205 *3.3 Analysis of the microbial communities*

206 3.3.1 Microbial diversity analysis

207 Principal Coordinate Analysis (PCoA) based on weighted-UniFrac distance matrix was used
208 to represent the differences of microbial communities between the inoculum (corresponding
209 to the 'bulks' at the beginning of each batch test) and the anode and bulk samples. Three clear
210 and significantly distinct clusters (*P*-value < 0.05) regardless to the electron donor are
211 observed in the PCoA plot (Fig. 2-a). Axis 1 represents 62.3% of the variance and allows to
212 distinguish inocula, biofilms and bulks while axis 2 represents 18.9% of the total variance. To
213 determine the differences in diversity between these three clusters and the electron donors, a
214 Shannon index was calculated (Fig. 2-b). The Shannon index gives access to the specific
215 diversity of each samples according to the number of species (species richness) and their
216 distribution (specific equitability). Among the three clusters (Fig. 2-a), a significant
217 difference was found between the Shannon indexes of the inocula, which had the highest
218 diversity (5.57±0.03), the bulks samples, having an average diversity (4.03±0.22), and the
219 anodic biofilms which had the lowest diversity (1.96±0.16) whatever the substrate. The
220 Butyrate-4 bulk sample had the lowest bulks' diversity (Fig. 2-a) which likely explained its
221 presence close to biofilm's cluster on PCoA plot (Fig. 2-b). Moreover, the amount of 16S
222 rRNA copies number, that is related to the cell number in each sample, is presented in Fig. 3.
223 Here, inocula samples contained the highest copy numbers with an average of
224 $2.95.10^{12}$±$3.69.10^{11}$ copies of the 16S rRNA gene. At the end of experiments in bulks a
225 significant decrease of the 16S rRNA copies number ($1.83.10^{11}$±$1.28.10^{11}$) 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-b). Concerning the biofilm samples, a number of $3.78.10^{10}\pm1.08.10^{10}$ 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.

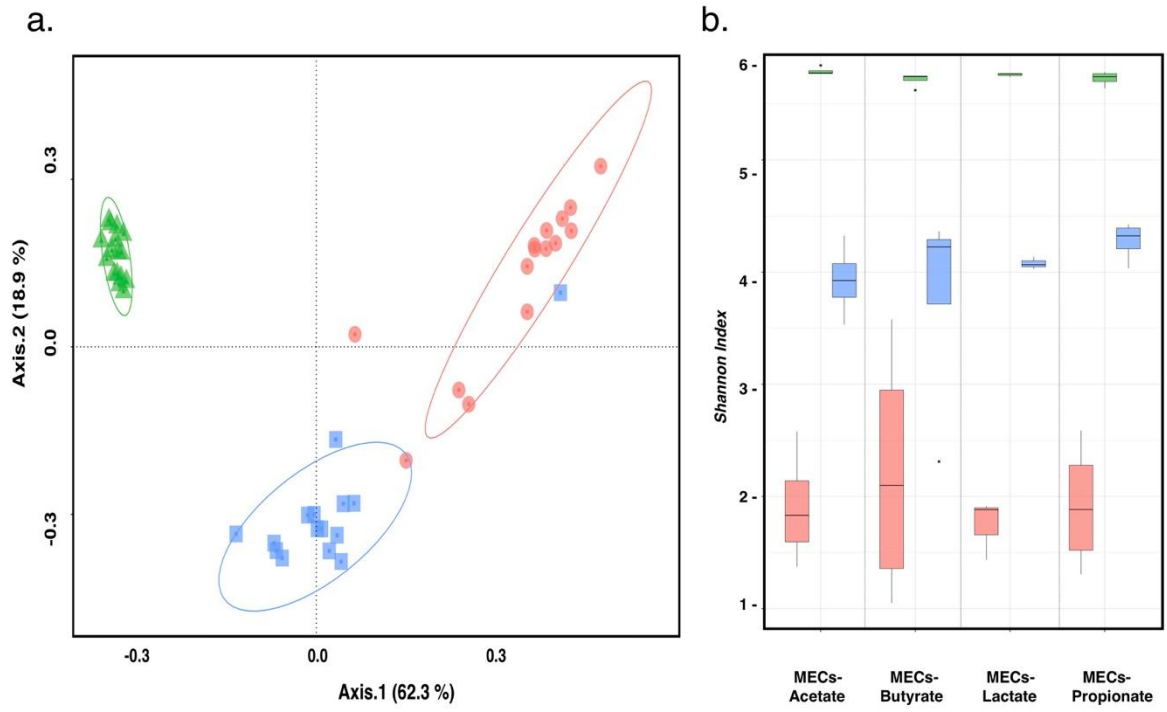
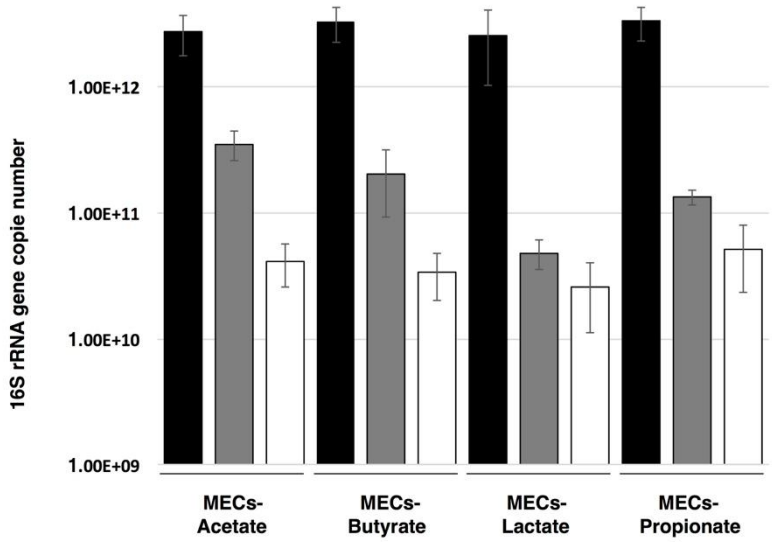


Fig. 2 a. Principal coordinate analysis (PCoA) based on weighted-UniFrac distance matrix showing the microbial distribution pattern for all substrates between inocula (▲ green triangles), bulks (■ blue squares) and biofilms (● red points) samples. Clusters were defined by significance difference calculated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



243

244 **Fig. 3** Average of qPCR measurements per sample types (Inocula ■, Bulks ■ and Biofilms □) in copie number
245 of 16S rRNA gene.

246

247 3.3.2 Anodic microbial communities according to the substrates

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

256 All biofilms whatever the substrate were dominated by members of the *Geobacteraceae*
257 family, representing 62.41% of biofilms' sequences (Fig. 4). The *Rickenelaceae* family was
258 also present in all biofilms samples at 10.89% and 12.61% in propionate-3 and acetate-3
259 MECs respectively. This family is represented by the *Blvii28* wastewater sludge group (OTU
260 9 & 54), known to be strict anaerobic fermenters [22]. 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 [23]. 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.

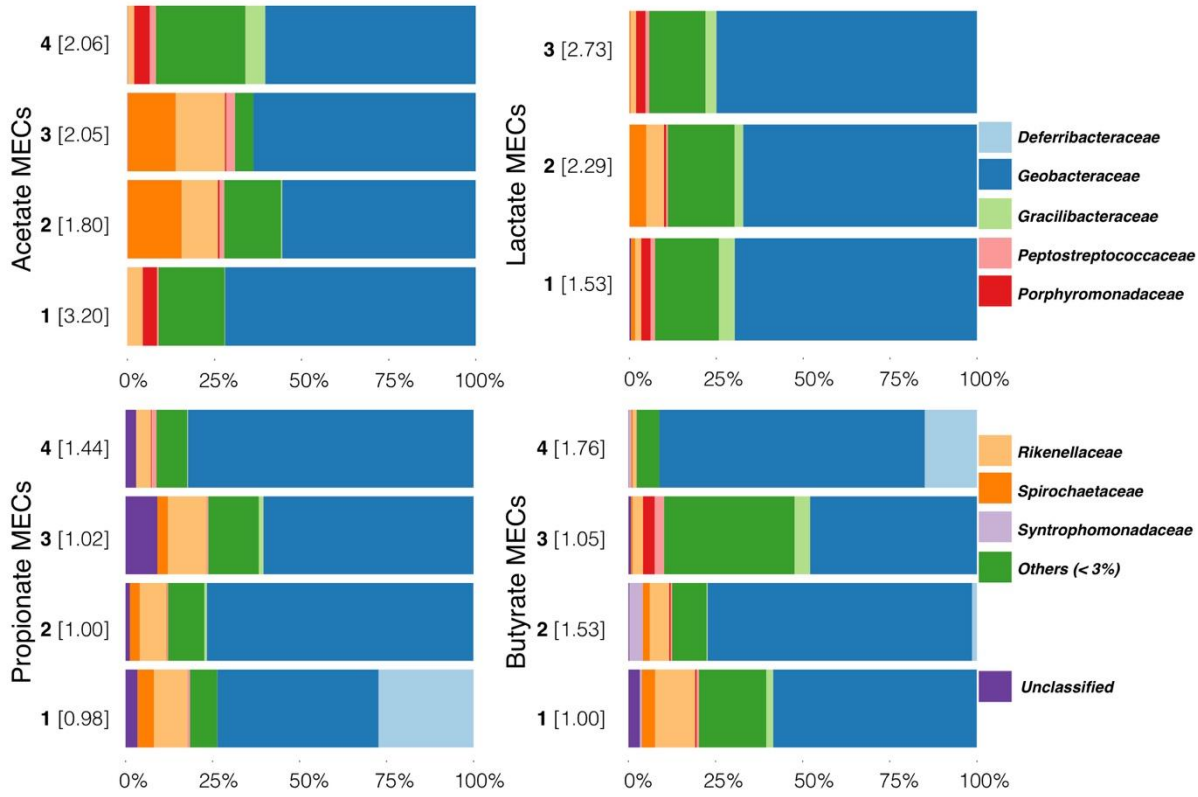


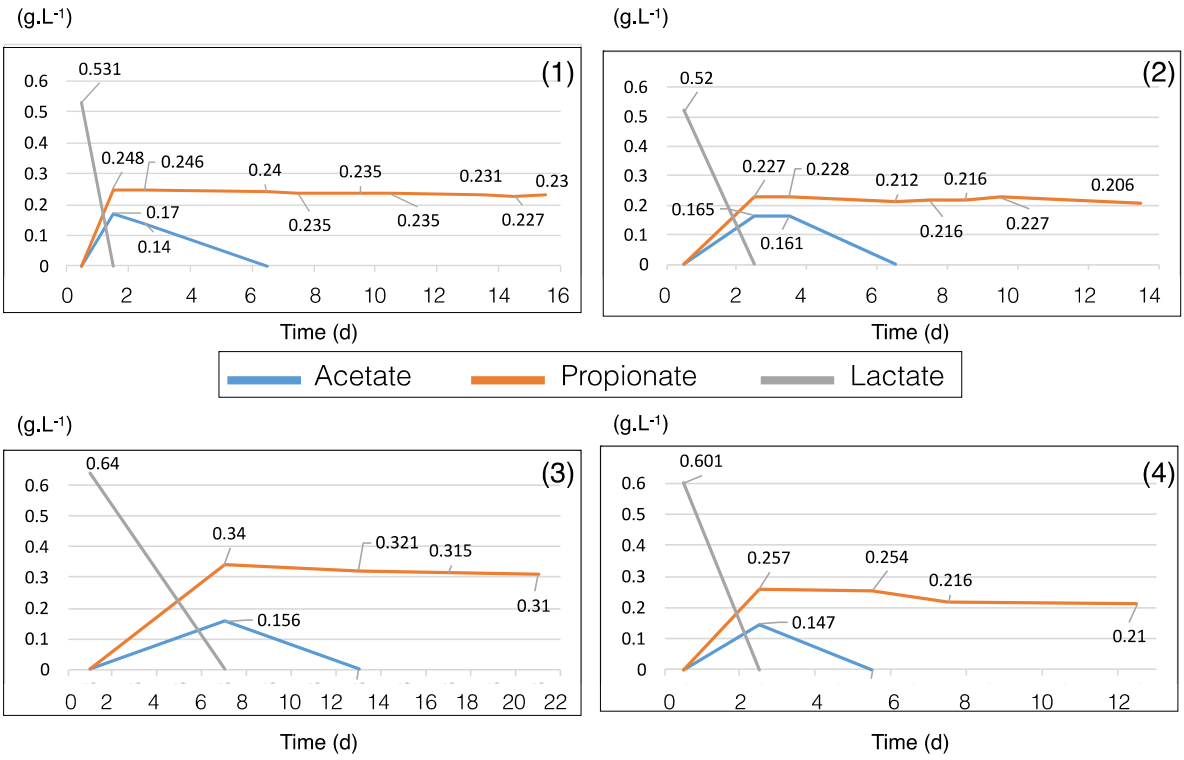
Fig. 4 Relative taxa abundances at Family level in anodic biofilms by MECs-type. Numbers (1-4) in bold are specific to the replicate (except Lactate-MEC 4). Numbers in square brackets correspond to the peak current density ($A.m^{-2}$) of the sample.

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

277 exoelectrogenesis [24]. For better understanding the metabolic pathways and ecological
278 interactions, the balance between *Geobacter* species was analysed. At this level, it showed a
279 specific relative distribution according to the substrate within *Geobacter* species (Fig. 6).
280 *Geobacter sulfurreducens* (OTU 1) was the main *Geobacter species* in acetate, lactate
281 and butyrate-fed MECs (except Butyrate-4) with a balance of 96.03 ± 6.1%, 95.84 ± 4.4% and
282 64.55 ± 30% respectively. This species was also present at 39.80% in Propionate-3 MEC.
283 *Geobacter toluenoxydans* (OTU 2) was dominant in propionate-fed MECs (except
284 Propionate-3) at 78.51 ± 18% and replicate-4 of butyrate-fed MEC (97.78%). *Geobacter*
285 *metallireducens* (OTU 5) was the second most abundant species in Propionate-1 and -3 MECs
286 (38.20 ± 2%) and was also present at 9.33% and 21.24% in Acetate-4 and Butyrate-3 MEC
287 respectively. *Geobacter pelophilus* (OTU 12) was dominant in Butyrate-1 sample (52.85%)
288 and second most abundant in Butyrate-3 sample (23.53%) and at 7.05% in Lactate-2 MEC.
289
290 These results suggest that when acetate was in solution as main substrate or co-product i.e.
291 Acetate- and Lactate-fed MECs (Fig. 5 & Fig. S-1), *G. sulfurreducens* predominated within
292 the *Geobacter* genus. Indeed, this EAB is a well-known electroactive microorganism able to
293 oxidize formate, H_2, lactate and acetate with the anode as sole terminal electron acceptor [25].
294 It could therefore be directly involved in acetate oxidation at the anode. *G. pelophilus* and *G.*
295 *metallireducens*, which are also able to convert acetate into current were found in a minority
296 in Acetate- and Lactate-fed MECs [26, 27]. Consequently, with regard to acetate as electron
297 donor, competitive relations likely took place for anode colonization within the *Geobacter*
298 genus. The competitive property of *G. sulfurreducens* leading to its predominance in the
299 ecosystem has also been observed in a synthetic consortium [28]. In lactate-fed MECs, acetate
300 was quickly oxidized (~4-6 days) while propionate was not degraded (Fig. 5). As observed in
301 Fig. 4, these biofilms were dominated by *Geobacteraceae* family where *G. sulfurreducens*

was predominant (Fig. 6). Since the latter is unable to oxidizing 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 [29]. 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* [27]. Moreover, the latter is also known to establish syntrophic relationships with other *Geobacter* species [30]. 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 [28]. 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*.



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323 **Fig. 5** Metabolite concentrations (g.L^{-1}) during lactate-fed MECs assays over time (d) according to the replicate
324 numbers (1-4).

325

326 3.3.4 Analysis of bacterial communities according to MEC performance

327 Within the same substrate and similar physicochemical conditions, differences in peak current
328 densities were observed (Fig. 4 & Fig. 6). It is therefore interesting to determine whether
329 these differences can be explained by the composition of the bacterial communities. By this
330 means, effective or ineffective species with respect to current densities can be determined.
331 Firstly, for acetate- and lactate-MECs, there was a difference of 56.25 and 56.04 % between
332 the highest and lowest performance in CDs respectively (Fig. 4). With these substrates, *G.*
333 *sulfurreducens* was dominant regardless of the CDs produced. Therefore, these differences
334 were probably due to other bacterial families. In Acetate-MECs, the most efficient reactor
335 (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 H_2 with CO_2 to produce acetate via the Wood-Ljungdahl (acetyl-CoA) pathway [31]. 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. 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 Proprionate-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. peluphilus*) [29].

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 [11, 32]. 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 [33]. 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/H_2) 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/H_2 or formate) such as *G. metallireducens* and consumers (i.e. EABs) such as *G. pelophilus* or *G. sulfurreducens* (Fig. 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 them into electricity [34]. A summary of the different possible routes can be seen in Schematic 1. The syntrophic pathway is less effective than the direct pathway, due to thermodynamic limitations to maintain low partial hydrogen pressure [35]. 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.

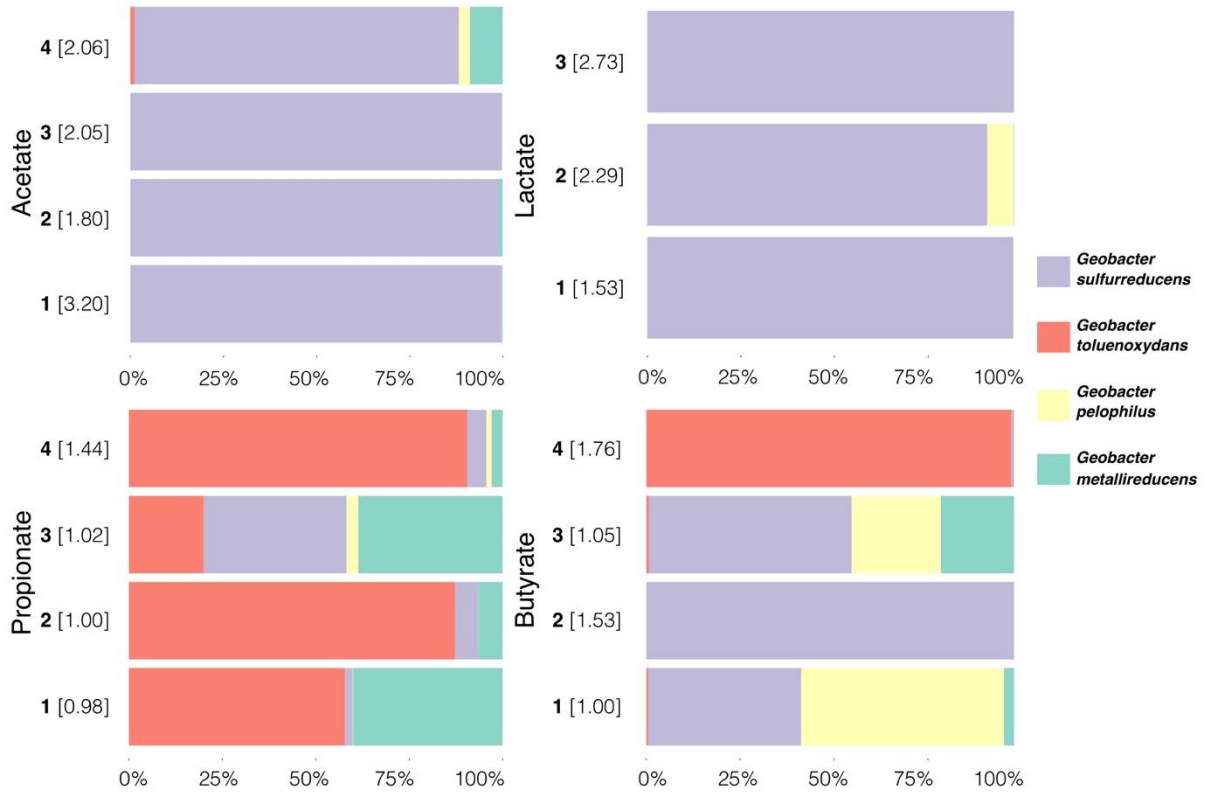
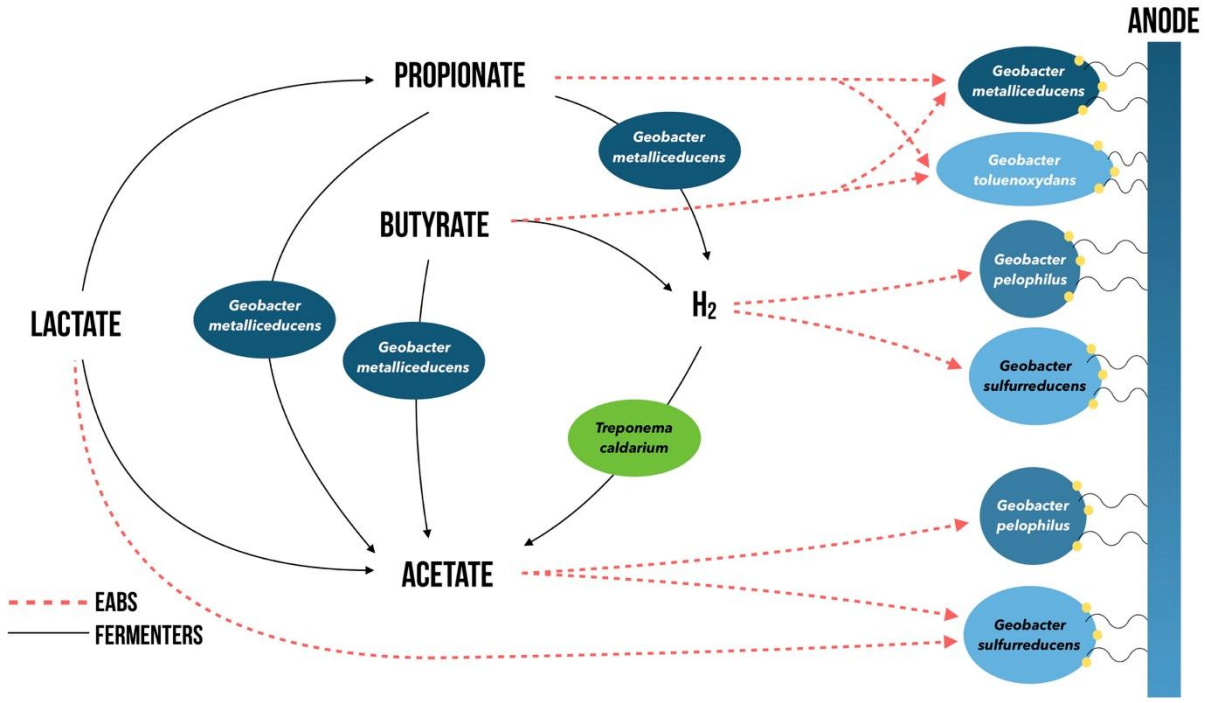


Fig. 6 Relative abundances of the main *Geobacter* species found in the anodic biofilms according to the fed substrate. The four *Geobacter sp*. represented 62.41% of total biofilm's sequences. Each *Geobacter sp.* is identified by one specific colour. Numbers (1-4) in bold are specific to the replicate (except Lactate-MEC 4). Numbers in brackets correspond to the maximum current density ($A.m^{-2}$) of the sample.



Schematic 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.

4 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.*

405 *metallireducens*) and consumers (e.g. *G. sulfurreducens* and *G. pelophilus*). Regarding the
406 link between bacterial communities and performances, *Treponema caldarium* appears to be
407 inefficient in the case of acetate oxidation, while *G. toluenoxydans* appears to be efficient for
408 the conversion of propionate to current by its ability to use this metabolite directly without
409 establishing syntrophy. Finally, these results allowing for the first time to make hypotheses of
410 the ecological relationships existing within electroactive consortia as well as the 'barrier
411 effect' that was probably caused by *G. sulfurreducens* and its low metabolic versatility
412 preventing propionate oxidation. Consequently, it would be interesting to better understand
413 the 'barrier effect' and the means to balance the electroactive ecosystems with propionate
414 effective-species to promote propionate oxidation with acetate in solution.

415 # 5 Acknowledgements

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