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▶ **To cite this version:**

Mélanie Pierra, Alessandro Carmona Martinez, Eric Trably, Jean-Jacques Godon, Nicolas Bernet. Specific and efficient electrochemical selection of Geoalkalibacter subterraneus and Desulfuromonas acetoxidans in high current-producing biofilms. Bioelectrochemistry, 2015, 106 (Pt A), pp.221-225. 10.1016/j.bioelechem.2015.02.003 . hal-02630636

HAL Id: hal-02630636
https://hal.inrae.fr/hal-02630636v1

Submitted on 7 Aug 2023

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1 **Specific and efficient electrochemical selection of *Geoalkalibacter*
2 *subterraneus* and *Desulfuromonas acetoxidans* in high current-producing
3 biofilms**
4
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11
12 **Abstract**
13 Two different saline sediments were used to inoculate potentiostatically controlled reactors (a
14 type of microbial bioelectrochemical system, BES) operated in saline conditions (35 g_{NaCl} l^{-1}).
15 Reactors were fed with acetate or a mixture of acetate and butyrate at two pH values: 7.0 or 5.5.
16 Electroactive biofilm formation lag-phase, maximum current density production and coulombic
17 efficiency were used to evaluate the overall performance of reactors. High current densities up
18 to 8.5 A m^{-2} were obtained using well-defined planar graphite electrodes. Additionally, biofilm
19 microbial communities were characterized by CE-SSCP and 454 pyrosequencing. As a result
20 of this procedure, two anode-respiring bacteria (ARB) always dominated the anodic biofilms:
21 *Geoalkalibacter subterraneus* and/ or *Desulfuromonas acetoxidans*. This suggests that a strong
22 electrochemically driven selection process imposed by the applied potential occurs in the BES
23 system. Moreover, the emergence of *Glk. subterraneus* in anodic biofilms significantly
24 contributes to broaden the spectrum of high current producing microorganisms
25 electrochemically isolated from environmental samples.
26
27 **Key words**: Microbial bioelectrochemical systems, 454 pyrosequencing, *Geoalkalibacter*
28 *subterraneus, Desulfuromonas acetoxidans*, saline wastewater
29

30 # 1 Introduction

31 Microorganisms embedded in electroactive biofilms convert organic matter into electrical
32 current through electron transfer mechanisms occurring in microbial bioelectrochemical
33 systems (BES). Such microorganisms are also commonly referred to as anode respiring bacteria
34 (ARB) due to their ability to transfer electrons to an electrode material via direct or indirect
35 electron transfer. ARB includes representatives of α, β, γ, δ-*Proteobacteria*, *Firmicutes*,
36 *Acidobacteria* and *Actinobacteria* which are able to transfer electrons to electrode materials [1].
37 While urban/domestic wastewater (WW) is a common source of microorganisms for the
38 development of electroactive biofilms in BESs, its application is consequently limited to the
39 treatment of domestic WW [2]. Hence, the selection of halophilic ARB is an essential
40 prerequisite for the treatment of saline WWs which represent more than 5% of the WWs
41 generated worldwide and considered a high risk effluent for soils, surfaces and groundwater
42 salinization [3]. Although it is well known that saline conditions increase the conductivity of
43 electrolyte solutions and thus facilitate the proton transport with the consequent increase of the
44 overall BES performance [3], this has been rarely tested. Liu *et al.* observed that an increase
45 from 1.7 to 6.8 g_{NaCl} l^{-1} induced an 85% increase in power densities in domestic wastewater-
46 based BESs [4]. More recently, Lefebvre *et al.* reported an increase of power densities by
47 progressively using higher concentrations of NaCl from 0.0 up to 20.0 g_{NaCl} l^{-1} [3]. However,
48 higher salinity concentrations proved to be detrimental to the overall performance of BESs in
49 those studies. Nevertheless, such cited studies were conducted with domestic WW as inoculum
50 and the ARB enriched were highly impacted at relatively low salinities. Accordingly, saline
51 sediments could be a better source of suitable halophilic ARB [5-7], an issue scarcely
52 investigated. Erable *et al.* obtained a current production of 2.5 A m^{-2} using a natural marine
53 biofilm on a graphite electrode polarized at -0.1 V vs. SCE and fed with 10mM acetate under
54 saline conditions (20 g_{NaCl} L^{-1}) [8]. Recently, Rousseau *et al.* observed a sharp increase in the
55 current density up to 85 A m^{-2} in a potentiostatically-controlled system (a type BES where the
56 electrodes' potential is controlled by a potentiostat). Their BES was operated under saline
57 conditions and fed with 40 mM acetate at 45 g_{NaCl} l^{-1} by developing an electroactive biofilm on
58 a porous carbon felt anode [9]. A decrease of the current production was observed at 60 g_{NaCl} l^{-}
59 ^{1} despite a significant performance (30 A m^{-2}). Unfortunately, little was known about the
60 microbial community involved. The present work aims at characterizing the microbial
61 community structure in high current-producing biofilms formed from saline sediments. We
62 investigated the strength of the selection pressure occurring in BESs under different
63 experimental conditions such as pH and electron donor.

64 # 2 Materials and methods

65 ## 2.1 General experimental procedure and description of Phase 1 and 2

66 The present work is part of a project aiming at feeding BESs with dead-end metabolites
67 resulting from the production of hydrogen by dark fermentation effluents. Consequently, the
68 tested pH values were acidic or neutral to compare conditions of dark fermentation and BESs,
69 respectively. In order to study the uptake of metabolites from a dark fermentation effluent, the
70 main organic acids were used here. To get close to characterisation of dark fermentation effluent
71 composition in saline conditions [14], acetate alone or a mixture of acetate and butyrate were
72 used as electron donors. In the case of reactors fed with a mixture of acetate and butyrate, a
73 complete conversion of butyrate into acetate was firstly observed. Then, acetate consumption
74 started. For all experimental units, biofilm sampling took place at the maximum current density.
75 Therefore, no complete substrate removal was observed (data not shown). Experiments were
76 divided in two phases. Phase 1 focused on the effects of pH and substrates on biofilm formation

77 and performance. Thus, a set of experiments was carried out using one saline sediment as
78 inoculum (Inoculum 1) in potentiostatically-controlled reactors (hereafter, reactors) operated
79 under different experimental conditions, *i.e.*: pH 7.0 or 5.5 and acetate only or acetate/butyrate
80 as carbon sources (please see Table 1). Once an experimental protocol was established in the
81 laboratory, a second set of experiments was conducted. This series, called Phase 2, was focused
82 on the effect of inoculum on biofilm formation and performance. Therefore, two different saline
83 sediments (Inoculum 1 and 2) were tested under the best conditions to produce current identified
84 in Phase 1, *i.e.*: pH 7.0 and acetate only as carbon source. Finally, biofilms were analyzed at
85 the maximal current density production to describe the microbial selection within the anodic
86 biofilm.

87 **2.2 Inocula source**

88 The two microbial inocula used here were sampled from two different locations: Inoculum 1
89 corresponded to sediments collected at the salt lake of Gruissan (France) and Inoculum 2
90 corresponded to sediments from a lagoon collecting the wastewaters of a salt factory (Salins de
91 Saint Martin, France). Both inocula presented similar physicochemical properties, *i.e.*, pH of
92 7.8±0.2; 36.1±3.5 gram of volatile solids per gram of sediments and a conductivity of 93.6±12.1
93 mS cm^{-1}. Those physicochemical similarities and operating conditions might explain why
94 regardless of the precedence of the inocula, similar ARB were highly enriched within the anodic
95 biofilms (Table 1).

96 **2.3 Bioelectrochemical set-up and operating conditions**

97 Reactor design and materials were exactly the same as those described by Carmona-Martínez
98 *et al.* [10]. In brief, experiments were carried out using electrochemical reactors under
99 potentiostatic control (VSP Bio-Logic SA) monitored with a computer (EC Laboratory v.10.1
100 software, Bio Logic SA). The set-up consisted of a working electrode (graphite plate), a SCE
101 reference electrode (KCl 3.0 M, +240 mV vs. SHE, Materials Mates, La Guilletière 38700
102 Sarcenas, France) and a counter electrode (platinum grid). The working electrode, *i.e.* the
103 anode, was a graphite planar electrode with the following dimensions: 2.5x2.5x0.2 cm
104 (C000440/15, Goodfellow SARL). To ensure an electrical connection, a 2 mm diameter and 12
105 cm long titanium rod (TI007910/13, Goodfellow SARL) was used. The counter electrode, *i.e.*
106 the cathode, was a Platinum Iridium grid (90%/10%) (Heraeus) cleaned by heating in a blue
107 flame as previously reported [11]. The anode potential was fixed at +200 mV vs. SCE during
108 all chronoamperometric (CA) growth. CA maximum current densities (j_{max} in A m^{-2}) of mature
109 microbial biofilms were calculated considering the total immersed electrode surface area and
110 regardless of the orientation of the working electrode towards the counter electrode (15 cm^{-2}).
111 Coulombic efficiencies (CE) were calculated for each experiment according to Call *et al.* [12].
112 The inoculum was added into the culture media (10% v/v for a final working volume of 400mL)
113 containing 50mM of MES buffer and mineral solution [13]. Reactors were fed with acetate (10
114 mM) or a mixture of acetate (5 mM) and butyrate (5 mM) as described in Table 1.
115 Concentrations in butyrate and acetate were determined according to Pierra *et al.* who reported
116 the fermentative production of acetate and butyrate in a saline environment [14]. All media
117 compositions were supplemented with 35 g_{NaCl} L^{-1} to simulate the conductivity and salinity
118 conditions commonly observed in sea water. The initial pH was adjusted to 5.5 or 7.0 using
119 NaOH (1 M). To ensure anaerobic conditions, reactors containing growth medium and
120 sediments were flushed with high purity N_2 (≥99.9999%) for at least 30 min. The final
121 composition of the gas phase was typically a mixture of N_2:98.56 ± 1.18% and O_2:1.41 ± 1.16%.
122 Such traces of oxygen might have caused the lag phase times observed in Fig. 1. Reactors were
123 incubated at 37°C since mesophilic conditions usually provide a favorable environment for fast

124 biofilm formation and optimum current density performance [15]. Acetic (C2) and butyric (C4)
125 acids were determined with a gas chromatograph (GC-3900 Varian) equipped with a flame
126 ionization detector, as previously described elsewhere [16].

127 **2.4 Microbial community analysis of electrochemically derived anodic biofilms**

128 Genomic DNA was extracted and purified using a previously described protocol [17]. Total
129 extracted DNA was purified using a QiAmp DNA microkit (Qiagen, Hilden, Germany). Then,
130 the region V3 of 16S rRNA genes were amplified using universal primers for bacteria (W49
131 and W104) according to Wéry *et al.* [18]. Each PCR mixture (50μL) contained 5μL of 10x Pfu
132 Turbo DNA buffer, 200 nMf of dNTP, 500 nMf of each primer, 2.5 U μl^{-1} of Pfu Turbo DNA
133 polymerase (Stratagene) and 10 ng of genomic DNA. Reactions were performed in a
134 Mastercycler thermal cycler (Eppendorf). The 16S rRNA genes were amplified as follows:
135 initial denaturing step at 94°C for 2 min, followed by 25 cycles performed at 94°C for 30 s,
136 61°C for 30 s and 72°C for 30 s, with a final elongation at 72°C for 10 min. Reactions were
137 stopped by cooling the mixture to 4°C. A capillary electrophoresis single-strand conformation
138 polymorphism (CE-SSCP) method was used for PCR products diversity characterization (see
139 Fig. S1). Samples were heat-denatured at 95°C for 5 min and re-cooled directly in ice for 5 min.
140 CE-SSCP electrophoresis was performed in an ABI Prism 3130 genetic analyzer (Applied
141 Biosystems) in 50 cm capillary tubes filled with 10% glycerol, conformation analysis polymer
142 and corresponding buffer (Applied Biosystems). Samples were eluted at 12kV and 32°C for 30
143 min, as described elsewhere [18]. CE-SSCP profiles were aligned with an internal standard
144 (ROX) to consider the inter-sample electrophoretic variability. CE-SSCP profiles were
145 normalized using the StatFingerprints library [19] in R software version 2.9.2 (R. Development
146 Core Team 2010). Simpson diversity index was calculated from SSCP profiles to estimate the
147 complexity of the community [20-22]. DNA samples were sequenced on a 454 GS-FLX
148 Titanium sequencer (Roche Life Sciences, USA) by the Research and Testing Laboratory
149 (Lubbock, USA). The sequences of the most abundant bacteria found in each biofilm were
150 deposited in the NCBI Genbank database under the following accession numbers: KF573509
151 to KF573519 for B1 to B9 biofilm respectively.

152 **3 Results and discussion**

153 **3.1 Chronoamperometric performance in terms of biofilm growth lag-phase, maximum**
154 **current density and coulombic efficiency**

155 **3.1.1 Phase 1, effect of pH and substrate on biofilm formation and performance**
156 In Phase 1, reactors were inoculated with saline lake sediments (Inoculum 1). The
157 representative chronoamperometric (CA) curves represented in Fig. 1 illustrate how lag phase
158 and maximum current density were calculated (see also Fig. S2). The Lag-phase for every
159 biofilm reported in Table 1 was determined taking into account the beginning of the exponential
160 increase in current density production. As depicted in Fig. 1, the lag-phase for each anode was
161 not identical. Notwithstanding, this difference might have been caused by the distinct
162 experimental parameters used here such as pH/substrate (phase 1) and inocula (phase 2).
163 Furthermore, each electroactive biofilm depicted a slightly different exponential growth.
164 Typically, shorter lag-phases led to shorter exponential growth phases (see Fig. S3).
165
166 -Please insert Figure 1 here-
167
168 By using the same inoculum source (Inoculum 1), two pH values and two different electron
169 donors (Table 1), the maximum current density (j_{max}) ranged from 1.9 to 4.2 A m^{-2}, for reactors

170 B1 to B4, respectively. The Coulombic Efficiency (CE) varied between 11 % to 70 % either
171 with a mixture of acetate and butyrate or acetate as sole carbon source, at pH 7.0 or 5.5.
172 Interestingly, when reactors were fed with a mixture of acetate and butyrate, a complete
173 conversion of butyrate into acetate was first observed before acetate consumption started (data
174 not shown). The low CE achieved is an indication of either other reactions than direct
175 conversion into current or the presence of internal electron loops [23]. Among those 4 reactors
176 (B1-4), the highest j_{max} (4.2 A m^{-2}), the highest CE (70 %) and the shortest lag phase (1.5 days)
177 were obtained at neutral pH and with acetate as the sole electron donor, as commonly reported
178 in the literature (see Fig. S4) [15, 24]. Hence, these conditions (pH 7.0 and acetate as carbon
179 source) were selected to perform further experiments in Phase 2.
180
181 -Please insert Table 1 here-
182

183 3.1.2 Phase 2, effect of inoculum on biofilm formation and performance

184 In Phase 2, five additional reactors were inoculated with sediments from a salt lake or from an
185 industrial salt-producing platform at pH 7.0 with acetate as carbon source (see Table 1). On
186 average, j_{max} values were 7.6±0.9 A m^{-2} and 5.0±1.1 A m^{-2} and CEs were 63±12 % and 76±5 %
187 with reactors inoculated with sediments from site 2 and site 1, respectively. Those results show
188 that experiments carried out in phase 2 outperformed those in phase 1 in terms of j_{max} and CE,
189 except for the case of B4 (pH 7.0, In 1, fed with acetate). This observation confirms that pH 7.0
190 and acetate as an electron donor are the most appropriate conditions tested in phase 1 for the
191 enrichment of anode-respiring bacteria (ARB) from saline sediments.
192 When added alone, acetate proved to be an effective electron donor for bacteria that compose
193 the electroactive biofilm enriched from sediments in terms of lag-phase, j_{max} and CE (see Table
194 1). This observation is consistent with previous studies in which environmental samples,
195 regardless of the origin of the inoculum, have been tested under well potentiostatically
196 controlled conditions as a source to develop high current-producing biofilms [5, 6, 25]. Freguia
197 *et al.* fed BESs with a mixture of more than 7 different volatile fatty acids (VFAs). They tested
198 the harvest of electrons by an electroactive biofilm from different substrates. They found out
199 that acetic and propionic acids were the preferential electron donors for ARB enriched in those
200 microbial biofilms [26]. Consistently, an enriched electroactive biofilm in a BES fed with
201 acetate generated 66% more power density than a BES fed with butyrate [4].
202 In the present work, the overall performance was higher at pH 7.0 than at pH 5.5 in terms of
203 CE (see Table 1). For reactors fed with acetate, CEs were 70% and 12% at pH 7.0 and 5.5,
204 respectively. This is consistent with previous results in which electroactive biofilms showed a
205 better performance in terms of CE at neutral pH [24].
206 As shown in this study, the pH is an important parameter for microbial electron transfer. When
207 substrate oxidation occurs at the anodic microbial biofilm, protons are produced and released
208 to the media. From there, they migrate to the cathode where they react with the electrons from
209 the external driven flow to finally produce hydrogen. A pH imbalance in both anode and
210 cathode environments could cause irreversible anodic biofilm degradation and thus severely
211 affect the reduction reaction [24].

212 3.2 Microbial community analysis of anodic biofilms

213 3.2.1 Electrochemically driven selection of anode-respiring bacteria

214 The microbial community of electroactive biofilms resulted in simple communities structures
215 with an average Simpson index of 0.86±0.06. In comparison, the inoculum was more diverse
216 with a Simpson diversity index of 0.98±0.00. This confirms the high selection of ARB which
217 occurred within the anodic biofilms in the present study. Such strong and specific selection of

218 ARB species from environmental samples has been extensively and almost exclusively reported
219 in previous studies using WW as a source of inoculum [5, 26, 27]. In the present study, the
220 analysis of 16S ribosomal DNA genes by 454 pyrosequencing showed a pronounced dominance
221 of bacteria from the δ subgroup of *Proteobacteria* with 85% to 97% of the 16S rDNA sequences
222 from the anodic biofilms belonging to this group. Interestingly, the most abundant ARB species
223 found in all electroactive biofilms were closely related and were up to 97% DNA similar to
224 either *Desulfuromonas acetoxidans* or *Geoalkalibacter subterraneus* in all reactors (see Table
225 1).
226 Of special interest is the presence of *Glk. subterraneus*, an ARB that has only been reported in
227 a recent study which showed that *Glk. subterraneus* dominated electroactive biofilms derived
228 from environmental anaerobic samples [6]. Therefore, the present results are consistent with
229 previous studies showing a high ARB selection and a decrease of diversity [6]. On the other
230 side, multiple studies have reported that under such well-potentiostatically controlled
231 conditions, a high microbial enrichment is very likely [27-29]. This is conferred by the ability
232 of several *Deltaproteobacteria* to transfer electrons to an electrode material [30].
233 More precisely, such high microbial selection in the anodic biofilms resulted from several
234 specific and constant experimental conditions, *e.g.*: (i) the fixed anodic applied potential (+200
235 mV vs. SCE), (ii) the constant temperature (37°C), (iii) the neutral pH conditions (7.0), (iv) the
236 homogeneous mass transfer in the bulk medium due to continuous stirring, (iv) the use of a
237 synthetic medium containing a non fermentable substrate such as acetate or butyrate, (v) the
238 anaerobic conditions and more importantly (vi) the moderately halophilic conditions (35 g/L
239 NaCl).
240 In the literature, several examples can be found where such a high microbial selection has
241 occurred. For instance, in a recent work conducted under very similar experimental conditions,
242 Miceli *et al.* used diverse environmental samples to enrich (~90%) several putative anode-
243 respiring bacteria (including *Geobacter* spp. and *Geoalkalibacter* spp., among others) that
244 converted acetate to current densities higher than 1 A/m^2 [6]. Consistently with the work of
245 Miceli *et al.*, Yates *et al.* (2012) obtained highly enriched electroactive biofilms from different
246 municipal wastewaters [28]. Biofilms were mainly composed of *Geobacter* spp. (≥80%), a
247 well-known ARB capable of producing high current densities and strongly attach to electrodes
248 via the formation of ≥50µm thick biofilms [31]. Similarly, Harnisch *et al.* demonstrated with
249 flow-cytometry that besides the high microbial diversity of wastewater as inoculum, *Geobacter*
250 *sulfurreducens* can be systematically enriched as an ARB by employing well potentiostatically
251 controlled conditions similar to those used in the present work [27].
252 Although a high selection of only a few ARB occurred in the present study, this might not
253 always be desirable, especially when the system is intended to treat real effluents like saline
254 WWs. If such WWs are the target of a treatment process by an ARB biofilm based technology,
255 then a more diverse microbial community would probably show higher adaptation and
256 resistance to the usual changing operating conditions observed in real WW treatment.
257 Among the δ-*Proteobacteria*, many species, including *Desulfuromonas* spp. and
258 *Geoalkalibacter* spp., are able to oxidize organic compounds and reduce insoluble Fe(III)
259 oxides at the same time [5, 32]. The present study shows that the enriched ARB effectively used
260 the electrode material as an electron acceptor, as they do in a natural environment when using
261 other insoluble final electron acceptors such as iron or manganese oxides [33]. Consequently,
262 it can be proposed that for the ARB found here, especially for *Geoalkalibacter* spp., the capacity
263 to reduce insoluble electron acceptors (such as iron oxides) does confer the ability to transfer
264 electrons to electrode materials.

265 **3.2.2 *D. acetoxidans* selection in butyrate-acetate fed reactors and its implications for**
266 **the overall performance and the anodic microbial composition of BESs**
267 The simultaneous use of two substrates to feed a BES might have important implications in
268 terms of the overall performance of the system and the microbial composition of the anodic
269 community. It was previously shown that microbial composition and performance of
270 electroactive biofilms are highly dependent on the nature of the substrate. According to Chae
271 *et al.*, a higher CE was found when the anodic biofilm was enriched from an anaerobic digester
272 sludge fed with acetate (72%) than with butyrate (43%) at pH 7.0 [26]. Those results are
273 consistent with the results presented here at neutral pH fed with acetate and with an acetate and
274 butyrate mix (Table 1). Moreover, in Freguia *et al.*, the microbial composition of the anodic
275 biofilm was also more diverse. In butyrate-fed reactors, there was a predominance of α, β and
276 δ–*Proteobacteria* and *Firmicutes*, with a majority of β-*Proteobacteria*. In acetate-fed reactors,
277 there was a predominance of α, β and δ *Proteobacteria* with a majority of β and δ-
278 *Proteobacteria* [26]. Therefore, in the study by Freguia *et al.* [26], a lack of *Geobacteraceae* in
279 the butyrate fed-system suggests that this group of microorganisms is not able to harvest
280 electrons from butyrate oxidation.
281 Interestingly, *Glk. subterraneus* is able to oxidize butyrate whereas *D. acetoxidans* is not [32,
282 33]. The emergence of *D. acetoxidans* in acetate-butyrate fed reactors (B1 and B2 in Table 1)
283 was likely favoured by the conversion of butyrate into acetate that occurred before current
284 production took place (data not shown). In addition, *D. acetoxidans* is an ARB that was firstly
285 isolated from marine sediments and is known to grow anaerobically by oxidizing acetate with
286 the reduction of elemental sulphur or Fe(III) [32]. Additionally, an organism close to *D.*
287 *acetoxidans* was repeatedly identified in enriched anodes by Bond *et al.* [5]. In their work, a
288 pure culture of *D. acetoxidans* provided a current density of 0.157 A m^{-2} on a graphite electrode
289 poised at +200 mV vs. AgAgCl.

290 **3.2.3 Selection of *Glk. subterraneus* as an anode-respiring bacterium capable of**
291 **producing high current densities**
292 Whereas *D. acetoxidans* has been well described in the literature as an ARB [6, 23], members
293 of the *Geoalkalibacter* genus have only recently been found in electroactive biofilms. Only one
294 study so far reported *Glk. subterraneus* as a dominant ARB in the population of electroactive
295 microbial biofilms enriched from shoreline and mangrove sediments [6]. Biofilms highly
296 enriched in *Geoalkalibacter* sp. reported by Miceli *et al.* reached a current density ranging from
297 3.87 to 8.73 A m^{-2} after a first CA growth cycle on a graphite electrode polarized at -0.3 V vs.
298 Ag/AgCl [6]. Our results are analogous even if applied potentials differ. Such similarities in
299 terms of j_{max} are likely due to the saline conditions used in both cases (from 20 to 35 g_{NaCl} L^{-1})
300 in which the inocula grew in both studies. Besides the high anodic microbial selection of *Glk.*
301 *subterraneus* that occurred in anodic biofilms under saline conditions, Miceli *et al.*'s work and
302 the present work have shown that it is indeed possible to obtain high conversion of organic
303 matter into current even under such saline conditions, usually considered to inhibit bacterial
304 metabolism (j_{max} >4 A m^{-2}).
305 Additionally, the recent electrochemical and microscopic characterization of pure cultures of
306 *Glk. subterraneus* provides further information to explain the appearance of such ARB in the
307 anodic biofilms of the present study [10, 34]. Moreover, *Glk. subterraneus* is considered as
308 either alkalitolerant or alkaliphilic (here the pH of the inocula was 7.8±0.2) and is able to use a
309 wide panel of substrates as electron donors such as acetate and ethanol [33]. Furthermore, *Glk.*
310 *subterraneus* belongs to the *Geobacteraceae* family (90% of similarity). This similarity
311 between this ARB and the well-known ARB *Glk. subterraneus* in terms of current density
312 production, electron transfer mechanism and biofilm formation might explain the significant
313 performance of *Glk. subterraneus* [10].

314 **4 Conclusions**

315 In summary, among all conditions tested, *D. acetoxidans* and *Glk. subterraneus* were the main
316 dominant anode-respiring bacteria in all anodic electroactive biofilms, alone or together. This
317 reveals a high selectivity of the method used, very likely due to the saline conditions, neutral
318 pH, readily degradable substrate and applied potential, among others. Moreover, efficient
319 electroactive biofilms were obtained in terms of significantly high current densities that reached
320 up to 8.5 A m^{-2}. Hence, a high substrate conversion with coulombic efficiency up to 83% was
321 obtained. The later clearly indicates that most of the substrate was effectively converted into
322 current.

323 **5 Acknowledgements**

324 This research was financed by the French National Research Agency (ANR-09-BioE-10
325 DéfiH12). The authors gratefully acknowledge M. Abouakil and A. Bonnafous for their
326 technical assistance with the microbial analysis of anodic biofilms and K. Milferstedt for
327 critically reading the manuscript.

328

329

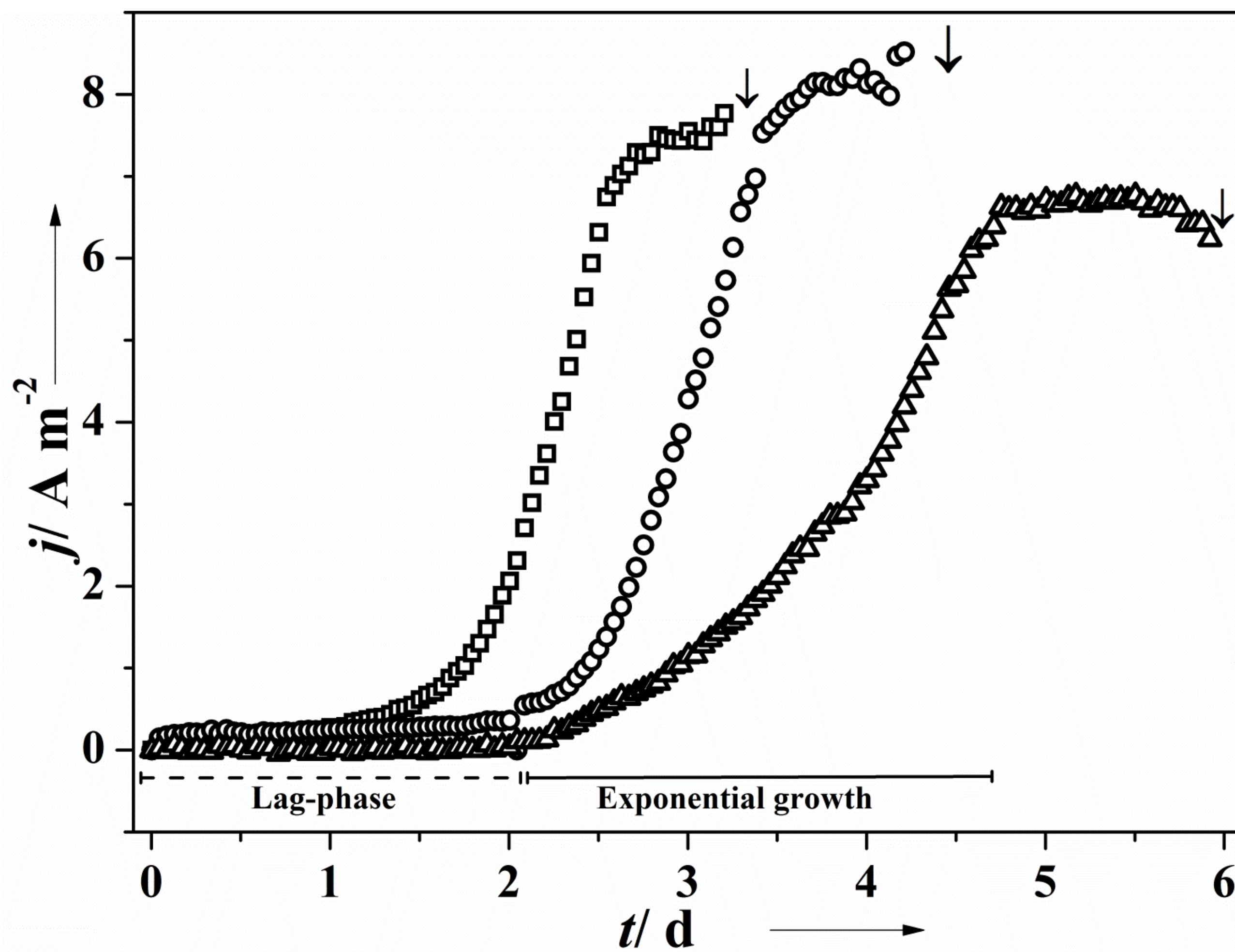
330 **Table 1** Performance of electrochemically derived biofilms grown under saline conditions

Experimental conditions*					Simpson diversity index	Lag phase/ days	j_{max}/ A m^{-2}	CE/ %	% Main dominant species in the biofilm (closest phylogenetical known sequences identified)†
B.	**pH**	**In.**	**Ac.**	**Bu.**					
					Phase 1, effect of pH and substrate on biofilm formation and performance				
B1	7.0	1	Yes	Yes	0.75	2.9	1.9	11	*D. acetoxidans* (86%)‡
B2	5.5	1	Yes	Yes	0.90	9.4	2.2	21	*D. acetoxidans* (98%)
B3	5.5	1	Yes	No	0.84	8.7	2.7	12	*D. acetoxidans* (99%)
B4	7.0	1	Yes	No	0.90	1.5	4.2	70	*Glk. subterraneus* (39%) *D. acetoxidans* (47%)
					Phase 2, effect of inoculum on biofilm formation and performance				
B5	7.0	1	Yes	No	0.87	2.2	4.5	80	*Glk. subterraneus* (95%)
B6	7.0	1	Yes	No	0.81	1.8	6.3	78	*Glk. subterraneus* (99%)
B7	7.0	2	Yes	No	0.93	1.9	6.7	60	*Glk. subterraneus* (73%)
B8	7.0	2	Yes	No	0.91	0.3	7.7	53	*Glk. subterraneus* (20%) *Desulfuromonas* spp. (58%)
B9	7.0	2	Yes	No	0.76	1.9	8.5	77	*Glk. subterraneus* (91%)

331 Notes: *B.: Biofilm, In.: inoculum type, Ac: acetate and Bu.: butyrate. †Names in *italics* correspond to
332 the closest phylogenetical known sequence based on the percentage of identity of dominant species
333 (>20% of total sequences). ‡Numbers in parentheses represent the relative abundance obtained from
334 454 pyrosequencing analysis.

335

336 **Fig. 1:** Representative chronoamperometric batch cycles of electrochemically derived biofilms
337 B7 (Δ), B8 (□) and B9 (○). Arrows indicate anodic biofilm collected for microbial analysis.



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