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Specific and efficient electrochemical selection of *Geoalkalibacter* 1

subterraneus and Desulfuromonas acetoxidans in high current-producing 2

- 3 biofilms
- 4 Mélanie Pierra, Alessandro A. Carmona-Martínez, Eric Trably, Jean-Jacques Godon, Nicolas Bernet*

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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).
- Reactors were fed with acetate or a mixture of acetate and butyrate at two pH values: 7.0 or 5.5. 15
- Electroactive biofilm formation lag-phase, maximum current density production and coulombic 16
- 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
- microbial communities were characterized by CE-SSCP and 454 pyrosequencing. As a result 19
- 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
- system. Moreover, the emergence of Glk. subterraneus in anodic biofilms significantly 23
- 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

Microorganisms embedded in electroactive biofilms convert organic matter into electrical 31 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 treatment of domestic WW [2]. Hence, the selection of halophilic ARB is an essential 39 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 from 1.7 to 6.8 g_{NaCl} l⁻¹ induced an 85% increase in power densities in domestic wastewater-45 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} 1⁻¹ [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 investigated. Erable et al. obtained a current production of 2.5 A m⁻² using a natural marine 52 biofilm on a graphite electrode polarized at -0.1 V vs. SCE and fed with 10mM acetate under 53 saline conditions (20 g_{NaCl} L⁻¹) [8]. Recently, Rousseau *et al.* observed a sharp increase in the 54 current density up to 85 A m⁻² in a potentiostatically-controlled system (a type BES where the 55 electrodes' potential is controlled by a potentiostat). Their BES was operated under saline 56 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⁻ ¹ despite a significant performance (30 A m⁻²). Unfortunately, little was known about the 59 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 investigated the strength of the selection pressure occurring in BESs under different 62 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 resulting from the production of hydrogen by dark fermentation effluents. Consequently, the 67 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 $(j_{max} \text{ in A m}^{-2})$ 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 compositions were supplemented with 35 g_{NaCl} L⁻¹ to simulate the conductivity and salinity 117 conditions commonly observed in sea water. The initial pH was adjusted to 5.5 or 7.0 using 118 119 NaOH (1 M). To ensure anaerobic conditions, reactors containing growth medium and 120 sediments were flushed with high purity N₂ (\geq 99.9999%) for at least 30 min. The final composition of the gas phase was typically a mixture of N_2 :98.56 ± 1.18% and O_2 :1.41 ± 1.16%. 121 Such traces of oxygen might have caused the lag phase times observed in Fig. 1. Reactors were 122 123 incubated at 37°C since mesophilic conditions usually provide a favorable environment for fast biofilm formation and optimum current density performance [15]. Acetic (C2) and butyric (C4)
 acids were determined with a gas chromatograph (GC-3900 Varian) equipped with a flame

acids were determined with a gas chromatograph (GC-3900 Varianionization 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 extracted DNA was purified using a QiAmp DNA microkit (Qiagen, Hilden, Germany). Then, 129 130 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 Turbo DNA buffer, 200 nMf of dNTP, 500 nMf of each primer, 2.5 U µl⁻¹ of Pfu Turbo DNA 132 133 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 Biosystems) in 50 cm capillary tubes filled with 10% glycerol, conformation analysis polymer 141 and corresponding buffer (Applied Biosystems). Samples were eluted at 12kV and 32°C for 30 142 143 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 normalized using the StatFingerprints library [19] in R software version 2.9.2 (R. Development 145 146 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 Titanium sequencer (Roche Life Sciences, USA) by the Research and Testing Laboratory 148 149 (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

In Phase 1, reactors were inoculated with saline lake sediments (Inoculum 1). The 156 157 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). Furthermore, each electroactive biofilm depicted a slightly different exponential growth. 163 164 Typically, shorter lag-phases led to shorter exponential growth phases (see Fig. S3).

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- 167

- -Please insert Figure 1 here-
- By using the same inoculum source (Inoculum 1), two pH values and two different electron 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 with a mixture of acetate and butyrate or acetate as sole carbon source, at pH 7.0 or 5.5. 171 172 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) were obtained at neutral pH and with acetate as the sole electron donor, as commonly reported 177 178 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.

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- 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 industrial salt-producing platform at pH 7.0 with acetate as carbon source (see Table 1). On 185 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 % 186 with reactors inoculated with sediments from site 2 and site 1, respectively. Those results show 187 188 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 the electroactive biofilm enriched from sediments in terms of lag-phase, jmax and CE (see Table 193 194 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].

In the present work, the overall performance was higher at pH 7.0 than at pH 5.5 in terms of CE (see Table 1). For reactors fed with acetate, CEs were 70% and 12% at pH 7.0 and 5.5, respectively. This is consistent with previous results in which electroactive biofilms showed a better performance in terms of CE at neutral pH [24].

As shown in this study, the pH is an important parameter for microbial electron transfer. When substrate oxidation occurs at the anodic microbial biofilm, protons are produced and released to the media. From there, they migrate to the cathode where they react with the electrons from the external driven flow to finally produce hydrogen. A pH imbalance in both anode and cathode environments could cause irreversible anodic biofilm degradation and thus severely

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

The microbial community of electroactive biofilms resulted in simple communities structures with an average Simpson index of 0.86 ± 0.06 . In comparison, the inoculum was more diverse

- with an average simpson index of 0.00 ± 0.00 . In comparison, the inocuration 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
- in previous studies using WW as a source of inoculum [5, 26, 27]. In the present study, the
- analysis of 16S ribosomal DNA genes by 454 pyrosequencing showed a pronounced dominance
- of bacteria from the δ subgroup of *Proteobacteria* with 85% to 97% of the 16S rDNA sequences
- from the anodic biofilms belonging to this group. Interestingly, the most abundant ARB species
- found in all electroactive biofilms were closely related and were up to 97% DNA similar to
 either *Desulfuromonas acetoxidans* or *Geoalkalibacter subterraneus* in all reactors (see Table
 1).
- Of special interest is the presence of *Glk. subterraneus*, an ARB that has only been reported in a recent study which showed that *Glk. subterraneus* dominated electroactive biofilms derived from environmental anaerobic samples [6]. Therefore, the present results are consistent with previous studies showing a high ARB selection and a decrease of diversity [6]. On the other side, multiple studies have reported that under such well-potentiostatically controlled conditions, a high microbial enrichment is very likely [27-29]. This is conferred by the ability 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
- specific and constant experimental conditions, *e.g.*: (i) the fixed anodic applied potential (+200
- 235 mV vs. SCE), (ii) the constant temperature $(37^{\circ}C)$, (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
- synthetic medium containing a non fermentable substrate such as acetate or butyrate, (v) the
 anaerobic conditions and more importantly (vi) the moderately halophilic conditions (35 g/L
- 239 NaCl).
- In the literature, several examples can be found where such a high microbial selection has occurred. For instance, in a recent work conducted under very similar experimental conditions,
- 241 becurred. 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-
- 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. (\geq 80%), a 247 well-known ARB capable of producing high current densities and strongly attach to electrodes
- 248 via the formation of \geq 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
- controlled conditions similar to those used in the present work [27].
- Although a high selection of only a few ARB occurred in the present study, this might not always be desirable, especially when the system is intended to treat real effluents like saline WWs. If such WWs are the target of a treatment process by an ARB biofilm based technology, then a more diverse microbial community would probably show higher adaptation and resistance to the usual changing operating conditions observed in real WW treatment.
- Among the δ -*Proteobacteria*, many species, including *Desulfuromonas* spp. and *Geoalkalibacter* spp., are able to oxidize organic compounds and reduce insoluble Fe(III) oxides at the same time [5, 32]. The present study shows that the enriched ARB effectively used the electrode material as an electron acceptor, as they do in a natural environment when using
- other insoluble final electron acceptors such as iron or manganese oxides [33]. Consequently,
- it can be proposed that for the ARB found here, especially for *Geoalkalibacter* spp., the capacity
- to reduce insoluble electron acceptors (such as iron oxides) does confer the ability to transfer
- 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 terms of the overall performance of the system and the microbial composition of the anodic 268 269 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 isolated from marine sediments and is known to grow anaerobically by oxidizing acetate with 285 286 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.

2903.2.3Selection of *Glk. subterraneus* as an anode-respiring bacterium capable of291producing 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*. subterraneus that occurred in anodic biofilms under saline conditions. Miceli et al.'s work and 301 302 the present work have shown that it is indeed possible to obtain high conversion of organic matter into current even under such saline conditions, usually considered to inhibit bacterial 303 304 metabolism ($j_{max} > 4 \text{ A m}^{-2}$).

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 dominant anode-respiring bacteria in all anodic electroactive biofilms, alone or together. This 316 317 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 up to 8.5 A m⁻². Hence, a high substrate conversion with coulombic efficiency up to 83% was 320 321 obtained. The later clearly indicates that most of the substrate was effectively converted into 322 current.

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329

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

Experimental conditions*					Simpson	Lag	j _{max} /	CE/	% Main dominant species in the
В.	pН	In.	Ac.	Bu.	 diversity index 	phase/ days	A m ⁻²	%	biofilm (closest phylogenetical known sequences identified) [†]
					Phase 1, effect of pH and substrate on biofilm formation and performance				
B 1	7.0	1	Yes	Yes	0.75	2.9	1.9	11	D. acetoxidans $(86\%)^{\ddagger}$
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

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.

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Fig. 1: Representative chronoamperometric batch cycles of electrochemically derived biofilms B7 (Δ), B8 (\Box) and B9 (\circ). Arrows indicate anodic biofilm collected for microbial analysis.



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340 6 References

- [1] H. Liu, H. Hu, J. Chignell, Y. Fan, Microbial electrolysis: novel technology for hydrogen
 production from biomass, Biofuels, 1 (2009) 129-142.
- 343 [2] O. Lefebvre, A. Uzabiaga, I. Chang, B.-H. Kim, H. Ng, Microbial fuel cells for energy self-
- sufficient domestic wastewater treatment a review and discussion from energetic
 consideration, Applied Microbiology and Biotechnology, 89 (2011) 259-270.
- [3] O. Lefebvre, Z. Tan, S. Kharkwal, H.Y. Ng, Effect of increasing anodic NaCl concentration
 on microbial fuel cell performance, Bioresource Technology, 112 (2012) 336-340.
- [4] H. Liu, S. Cheng, B.E. Logan, Power generation in fed-batch microbial fuel cells as a
 function of ionic strength, temperature, and reactor configuration, Environmental Science and
 Technology, 39 (2005) 5488-5493.
- [5] D.R. Bond, D.E. Holmes, L.M. Tender, D.R. Lovley, Electrode-Reducing Microorganisms
 That Harvest Energy from Marine Sediments, Science, 295 (2002) 483-485.
- 353 [6] J.F. Miceli, P. Parameswaran, D.-W. Kang, R. Krajmalnik-Brown, C.I. Torres, Enrichment
- and Analysis of Anode-Respiring Bacteria from Diverse Anaerobic Inocula, Environmental
 Science & Technology, 46 (2012) 10349-10355.
- 356 [7] D.E. Holmes, J.S. Nicoll, D.R. Bond, D.R. Lovley, Potential Role of a Novel
- 357 Psychrotolerant Member of the Family Geobacteraceae, Geopsychrobacter electrodiphilus gen.
- nov., sp. nov., in Electricity Production by a Marine Sediment Fuel Cell, Applied and
 Environmental Microbiology, 70 (2004) 6023-6030.
- [8] B. Erable, M.-A. Roncato, W. Achouak, A. Bergel, Sampling Natural Biofilms: A New
 Route to Build Efficient Microbial Anodes, Environmental Science & Technology, 43 (2009)
 3194-3199.
- 363 [9] R. Rousseau, X. Dominguez-Benetton, M.-L. Délia, A. Bergel, Microbial bioanodes with 364 high salinity tolerance for microbial fuel cells and microbial electrolysis cells, Electrochemistry 265 Communications 22 (2012) 1.4
- 365 Communications, 33 (2013) 1-4.
- [10] A.A. Carmona-Martínez, M. Pierra, E. Trably, N. Bernet, High current density via direct
 electron transfer by the halophilic anode respiring bacterium Geoalkalibacter subterraneus,
 Physical Chemistry Chemical Physics, 15 (2013) 19699-19707.
- [11] S.F. Ketep, A. Bergel, M. Bertrand, W. Achouak, E. Fourest, Lowering the applied
 potential during successive scratching/re-inoculation improves the performance of microbial
 anodes for microbial fuel cells, Bioresource Technology, 127 (2013) 448-455.
- 372 [12] D.F. Call, R.C. Wagner, B.E. Logan, Hydrogen Production by Geobacter Species and a
- Mixed Consortium in a Microbial Electrolysis Cell, Applied and Environmental Microbiology,
 75 (2009) 7579-7587.
- 375 [13] Y. Rafrafi, E. Trably, J. Hamelin, E. Latrille, I. Meynial-Salles, S. Benomar, M.-T. Giudici-
- 376 Orticoni, J.-P. Steyer, Sub-dominant bacteria as keystone species in microbial communities 377 producing bio-hydrogen, International Journal of Hydrogen Energy, 38 (2013) 4975–4985.
- [14] M. Pierra, E. Trably, J.-J. Godon, N. Bernet, Fermentative hydrogen production under
 moderate halophilic conditions, 39 (2013) 7508–7517.
- 380 [15] S.A. Patil, F. Harnisch, B. Kapadnis, U. Schröder, Electroactive mixed culture biofilms in
- microbial bioelectrochemical systems: The role of temperature for biofilm formation and
 performance, Biosensors and Bioelectronics, 26 (2010) 803-808.
- 383 [16] C.A. Aceves-Lara, E. Latrille, P. Buffiere, N. Bernet, J.-P. Steyer, Experimental 384 determination by principal component analysis of a reaction pathway of biohydrogen 385 production by anacrobia formantation. Chamical Engineering and Proceeding: Proceedings
- production by anaerobic fermentation, Chemical Engineering and Processing: Process
 Intensification, 47 (2008) 1968-1975.
- 387 [17] J.J. Godon, E. Zumstein, P. Dabert, F. Habouzit, R. Moletta, Molecular microbial diversity
- 388 of an anaerobic digestor as determined by small-subunit rDNA sequence analysis, Applied and 380 Environmental Microbiology 63 (1997) 2802 2813
- 389 Environmental Microbiology, 63 (1997) 2802-2813.

- [18] N. Wéry, V. Bru-Adan, C. Minervini, J.-P. Delgénes, L. Garrelly, J.-J. Godon, Dynamics
 of Legionella spp. and Bacterial Populations during the Proliferation of L. pneumophila in a
 Cooling Tower Facility, Applied and Environmental Microbiology, 74 (2008) 3030-3037.
- [19] R.J. Michelland, S. Dejean, S. Combes, L. Fortun-Lamothe, L. Cauquil, StatFingerprints:
- a friendly graphical interface program for processing and analysis of microbial fingerprint
 profiles, Molecular Ecology Resources, 9 (2009) 1359-1363.
- 396 [20] B. Haegeman, B. Sen, J.-J. Godon, J. Hamelin, Only Simpson Diversity can be Estimated
- Accurately from Microbial Community Fingerprints, Microbial Ecology, 68 (2014) 169-172.
- 398 [21] M. Quéméneur, J. Hamelin, A. Barakat, J.-P. Steyer, H. Carrère, E. Trably, Inhibition of
 399 fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures,
 400 International Journal of Hydrogen Energy, 37 (2012) 3150-3159.
- 401 [22] M. Quéméneur, J. Hamelin, E. Latrille, J.-P. Steyer, E. Trably, Functional versus
 402 phylogenetic fingerprint analyses for monitoring hydrogen-producing bacterial populations in
 403 dark fermentation cultures, International Journal of Hydrogen Energy, 36 (2011) 3870-3879.
- 404 [23] B.E. Logan, J.M. Regan, Electricity-producing bacterial communities in microbial fuel 405 cells, Trends in microbiology, 14 (2006) 512-518.
- 406 [24] S.A. Patil, F. Harnisch, C. Koch, T. Hübschmann, I. Fetzer, A.A. Carmona-Martínez, S.
- 407 Müller, U. Schröder, Electroactive mixed culture derived biofilms in microbial
- 408 bioelectrochemical systems: The role of pH on biofilm formation, performance and 409 composition, Bioresource Technology, 102 (2011) 9683-9690.
- [25] K.-J. Chae, M.-J. Choi, J.-W. Lee, K.-Y. Kim, I.S. Kim, Effect of different substrates on
 the performance, bacterial diversity, and bacterial viability in microbial fuel cells, Bioresource
 Technology, 100 (2009) 3518-3525.
- 412 Technology, 100 (2009) 5518-5525.
 413 [26] S. Freguia, E.H. Teh, N. Boon, K.M. Leung, J. Keller, K. Rabaey, Microbial fuel cells
- 414 operating on mixed fatty acids, Bioresource Technology, 101 (2010) 1233-1238.
- 415 [27] F. Harnisch, C. Koch, S.A. Patil, T. Hübschmann, S. Müller, U. Schröder, Revealing the 416 electrochemically driven selection in natural community derived microbial biofilms using flow-
- 417 cytometry, Energy and Environmental Science, 4 (2011) 1265-1267.
- 418 [28] M.D. Yates, P.D. Kiely, D.F. Call, H. Rismani-Yazdi, K. Bibby, J. Peccia, J.M. Regan,
- B.E. Logan, Convergent development of anodic bacterial communities in microbial fuel cells,
 ISME J, 6 (2012) 2002-2013.
- 421 [29] C.I. Torres, R. Krajmalnik-Brown, P. Parameswaran, A.K. Marcus, G. Wanger, Y.A.
- 422 Gorby, B.E. Rittmann, Selecting anode-respiring bacteria based on anode potential:
- 423 Phylogenetic, electrochemical, and microscopic characterization, Environmental Science and
- 424 Technology, 43 (2009) 9519-9524.
- 425 [30] S. Patil, C. Hägerhäll, L. Gorton, Electron transfer mechanisms between microorganisms
 426 and electrodes in bioelectrochemical systems, Bioanalytical Reviews, 4 (2012) 159-192.
- 427 [31] D.R. Lovley, T. Ueki, T. Zhang, N.S. Malvankar, P.M. Shrestha, K.A. Flanagan, M.
- 428 Aklujkar, J.E. Butler, L. Giloteaux, A.-E. Rotaru, D.E. Holmes, A.E. Franks, R. Orellana, C.
- 429 Risso, K.P. Nevin, K.P. Robert, Geobacter: The Microbe Electric's Physiology, Ecology, and
- 430 Practical Applications, in: Advances in Microbial Physiology, Academic Press, 2011, pp. 1-
- 431 100.
- [32] N. Pfennig, H. Biebl, Desulfuromonas acetoxidans gen. nov. and sp. nov., a new anaerobic,
 sulfur-reducing, acetate-oxidizing bacterium, Archives of Microbiology, 110 (1976) 3-12.
- 434 [33] A.C. Greene, B.K.C. Patel, S. Yacob, Geoalkalibacter subterraneus sp. nov., an anaerobic
- 435 Fe(III)- and Mn(IV)-reducing bacterium from a petroleum reservoir, and emended descriptions
- 436 of the family Desulfuromonadaceae and the genus Geoalkalibacter, International Journal of
- 437 Systematic and Evolutionary Microbiology, 59 (2009) 781-785.
- 438 [34] J.P. Badalamenti, R. Krajmalnik-Brown, C.I. Torres, Generation of High Current Densities
- 439 by Pure Cultures of Anode-Respiring Geoalkalibacter spp. under Alkaline and Saline
- 440 Conditions in Microbial Electrochemical Cells, mBio, 4 (2013) e00144-00113.