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1 **High current density via direct electron transfer by the halophilic anode respiring**
2 **bacterium *Geoalkalibacter subterraneus***

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6 **Keywords:** *Geoalkalibacter subterraneus*, Bioelectrochemical systems, Cyclic voltammetry,
7 Confocal Laser Scanning Microscopy, Direct electron transfer

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10 **Abstract**

11 In this study the characterization of *Geoalkalibacter subterraneus* is presented, a novel
12 halophilic anode respiring bacteria (ARB) previously selected and identified in a
13 potentiostically controlled bioelectrochemical system (BES) inoculated with sediments from a
14 salt plant. Pure culture electroactive biofilms of *Glk. subterraneus* were grown during
15 chronoamperometric batch experiments at a graphite electrode poised at +200 mV (vs. SCE)
16 with 10 mM acetate as electron donor. These biofilms exhibited the highest current density
17 ($4.68 \pm 0.54 \text{ A/m}^2$) reported on a planar material with a pure culture under saline conditions
18 (3.5% NaCl). To investigate possible anodic electron transfer (ET) mechanisms, cyclic
19 voltammetry (CV) of mature visible apparent reddish biofilms was performed under
20 bioelectrocatalytic substrate consumption (turnover) and in absence of substrate (non-
21 turnover). CV evidenced a well defined typical sigmoidal shape and a pair of clear redox
22 couples under turnover and non-turnover conditions, respectively. Moreover, the calculation
23 of their formal potentials indicated the presence of a common ET mechanism present in both
24 CV conditions between $-427.6 \pm 0.5 \text{ (} E_{f,2} \text{)}$ and $-364.8 \pm 4.5 \text{ mV (} E_{f,3} \text{)}$. Confocal laser scanning
25 microscopy inspection showed a biofilm structure composed of several layers of
26 metabolically active bacteria that spread all over the electrode material within a biofilm up to
27 $76 \pm 7 \mu\text{m}$ thick. Such value, high for the thickness normally reported in the literature for pure
28 culture electroactive bacteria justifies further investigations. Taken together, these results
29 suggest that *Glk. subterraneus* performs a direct ET mechanism in contact with the electrode
30 material. Furthermore, direct current generation from saline wastewaters significantly expands
31 the application of BESs.

32 1. Introduction

33 Bioelectrochemical systems (BESs) are a group of technologies derived from the bidirectional
34 electron transfer (ET) interactions between microorganisms and electrode materials¹. More
35 specifically: 1) the harvest of electrons from the oxidation of a substrate and their further
36 transfer to an electrode (*e.g.*, for the direct production of electricity²) and 2) the uptake of
37 electrons from the electrode material to reduce a substrate for the production of valuable
38 industrial products such as hydrogen gas³, sodium hydroxide or acetate⁴. Up to now, two main
39 microbial ET mechanisms have been extensively described: direct electron transfer (DET) and
40 mediated electron transfer (MET). Whereas in DET bacteria need to be in direct contact with
41 the electrode material to release/accept electrons via c-type cytochromes⁵, in MET bacteria
42 are capable of releasing/accepting electrons via self produced redox mediator molecules such
43 as flavins⁶ or phenazines⁷ (among others⁸⁻¹¹). Although microbial ET mechanisms in BESs
44 have been extensively studied in two model bacteria families such as *Geobacteraceae* and
45 *Shewanaellaceae*¹, there are many other bacteria capable of donating/accepting electrons
46 to/from an electrode (see Table S1).

47 While members of the *Geobacteraceae* family are capable of performing a DET mechanism,
48 with significant current densities (up to 9 A/m²)¹² and forming thick biofilms (about 50 μm¹³⁻
49 ¹⁸) in freshwater environments, *Shewanaellaceae* is capable of performing both DET and
50 MET mechanisms. Interestingly, *Shewanaellaceae* produces very low current densities¹⁹,
51 likely due to their limitation to form thin-monolayer biofilms on the electrode surface^{20, 21}.
52 Although their ET mechanisms are extensively understood, there is still a great interest to
53 identify and characterize other bacteria capable of forming electroactive biofilms and
54 producing significant current densities since they can aid to comprehend more precisely how
55 ET occurs through different microbial species^{22, 23}. Furthermore, direct current generation
56 from saline wastewaters as the ones produced in the sea-food, petroleum and leather
57 industries could significantly broad the application of BESs.

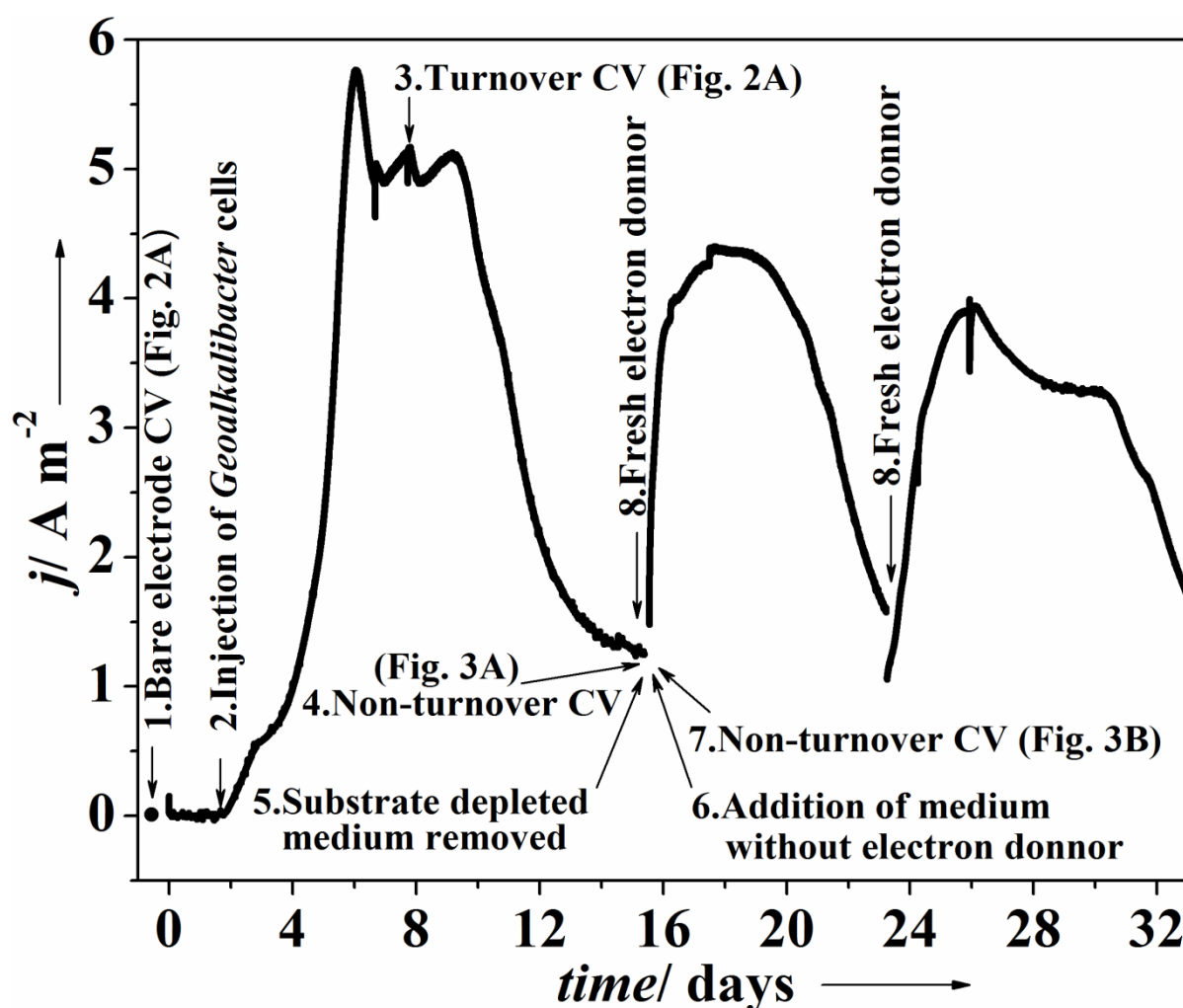
58 For the characterization of electroactive bacteria diverse techniques are available. These
59 techniques target practically all type of parameters, from architecture to electrochemistry of
60 biofilms²⁴. Here, the novel halophilic anode respiring bacterium (ARB) *Geoalkalibacter*
61 *subterraneus* (henceforth, *Glk. subterraneus*) is characterized. *Glk. subterraneus* was
62 previously selected and identified in a potentiostically controlled BES inoculated with
63 sediments from a salt plant²⁵. An electrochemical as well as a microscopic approach was
64 employed. Chronoamperometry was used to evaluate *Glk. subterraneus*'s maximum current

65 density production with intermittent measurements of its ET mechanism by cyclic
66 voltammetry and confocal laser scanning microscopy to analyze biofilm electrode coverage
67 and thickness.

68 2 Results and discussion

69 2.1 Bioelectrochemical biofilm formation of *Geoalkalibacter subterraneus* and 70 generation of high current density under saline conditions

71 Fig. 1 shows a representative chronoamperometric (CA) biofilm growth of *Glk. subterraneus*
72 on a graphite planar electrode at an applied potential of +200 mV (vs. SCE) with 10 mM
73 sodium acetate as the electron donor. For all CA biofilm growth experiments, strict anoxic
74 conditions were procured as described in the Experimental section and routinely tested by gas
75 chromatographic analysis since *Glk. subterraneus* preferentially grows under these
76 conditions²⁶.



77
78 **Fig. 1** Representative chronoamperometric fed-batch cycles of *Geoalkalibacter subterraneus*
79 biofilms grown on graphite planar electrodes (15 cm²); applied potential: +200 mV vs. SCE
80 (KCl 3.0 M).

81 As denoted in Fig. 1, approximately after 24 h of poisoning the working electrode immersed in
82 the growth medium, the electrochemical cell was inoculated and it took around 24 more hours
83 for biofilm growth to begin. This was illustrated as an exponential-like current production
84 trend, characteristic of ARB able to produce high currents and thick biofilms²⁷⁻²⁹ (see Table
85 S1). Fig. 1 shows that at day six of incubation, the current density peaked a maximum value
86 (j_{max}) of about 5.7 A/m². This value establishes the highest current density produced by an
87 ARB pure culture under saline conditions (3.5% NaCl)³⁰.

88 When comparing j_{max} by pure culture biofilms of *Glk.*, our results are consistent with previous
89 observations in our group for biofilms enriched with *Geoalkalibacter* in potentiostically
90 controlled BESs inoculated with sediments from a salt plant (j_{max} : 4.5 to 8.5 A/m²)³¹.
91 Furthermore, our results corroborate as well those of a recent publication by Miceli *et al.*,
92 (2012)³² showing *Geoalkalibacter* dominated biofilms derived from environmental anaerobic
93 samples (j_{max} : 4.2 and 8.9 A/m²). Similarly, Badalamenti *et al.* (2013)³⁰ reported the
94 electrochemical performance of *Glk. subterraneus* grown on graphite rod electrodes under
95 lower saline conditions (1.7% NaCl). They showed also a significant high j_{max} (3.3 A/m²) but
96 lower than the one reported here (5.7 A/m²). One reason why current densities reported here
97 were higher, could be the discrepancy between their and our experimental procedures (see
98 Table S2); *e.g.*: higher concentration of salt (therefore increasing the conductivity of the
99 electrolyte solution³³), more positive applied potential (hence increasing biofilm formation¹⁹)
100 and distance between working and counter electrodes due to BES architecture³⁴. Nevertheless,
101 in our experiments a lower concentration of electron donor (10 mM sodium acetate) generated
102 higher current densities. A finding not totally consistent with literature data, since it has been
103 extensively demonstrated that the current density augments by increasing the concentration of
104 substrate until saturation kinetics^{35,36}.

105 After the maximum current density was reached, a period of a stable current production was
106 observed at 4.68 ± 0.54 A/m² (when both reproducible replicates are considered for the
107 calculation, see Fig. S1). In between, CA was stopped and turnover CV was performed as
108 indicated in the Experimental section. Around day nine, CA showed a sudden current
109 decrease due to substrate exhaustion as confirmed at the end of the cycle by metabolite
110 analysis. The conversion of substrate into current as coulombic efficiency (CE) was assessed
111 by considering the ratio of electrons actually obtained from the substrate and the amount of
112 electrons theoretically available². Here, in semi-batch half-cell experiments, CE exceeded
113 100% (114 ± 14). This was likely due to oxidation of hydrogen produced at the cathode³⁷

114 since a significant percentage (oxidizable by *Glk. subterraneus*²⁶) was detected in the gas
115 phase of our experiments (e.g., the gas composition during CA at the maximum of current
116 production was CO₂ 16.6 %, H₂ 82.2 %, O₂ 1.05% and CH₄ 0.34 %). The focus of this study
117 was the characterization of *Glk. subterraneus* in terms of current output, electron transfer and
118 biofilm formation and not maximizing CE as would be typical for applied BESs such as
119 microbial fuel cells² or microbial electrolysis cells³.

120 In several studies where electroactive bacteria are characterized as a pure culture, it has been
121 usually found that in further batch CA cycles performed by media replenishment, the current
122 recovers or exceeds its previous value³⁸⁻⁴⁴. As seen in Fig. 1, the current density did not
123 recover its previous value after the substrate depleted media was replenished at the end of the
124 first and second CA semi-batch cycles. On the other side, the immediate production of current
125 can be considered a proof of bacteria's ability to convert the supplied substrate into current
126 instead of using it for biosynthesis⁴⁵. Therefore, this points out a possible direct electron
127 transfer (DET) mechanism²³.

128 A similar phenomenon where current density does not recover its previous value was also
129 observed elsewhere^{22, 30, 46-52}. A possible explanation could be that the presence of a substance
130 (or substances) produced in the previous CA cycle is required to achieve previous similar
131 performance, hence hindering the biofilm to produce as much current density as in the
132 previous CA cycle.

133 **2.1.1 Comparison of *Geoalkalibacter subterraneus* and *Geobacter sulfurreducens* in terms** 134 **of chronoamperometric current density production**

135 To conduct an accurate comparison of the performance of *Glk. subterraneus*, parallel
136 electrochemical experiments were carried out with the extensively well characterized^{12, 53-60}
137 model bacteria *Geobacter sulfurreducens* (hereafter, *Gb. sulfurreducens*). *Gb. sulfurreducens*
138 was employed here as an appropriate reference of an ARB in terms of j_{max} obtained during CA
139 experiments, analysis of its ET mechanism with cyclic voltammetry (CV) and biofilm
140 examination with the use of confocal laser scanning microscopy (CLSM) under similar
141 experimental conditions; i.e.: identical half-cell architecture and components, temperature, pH
142 and applied potential.

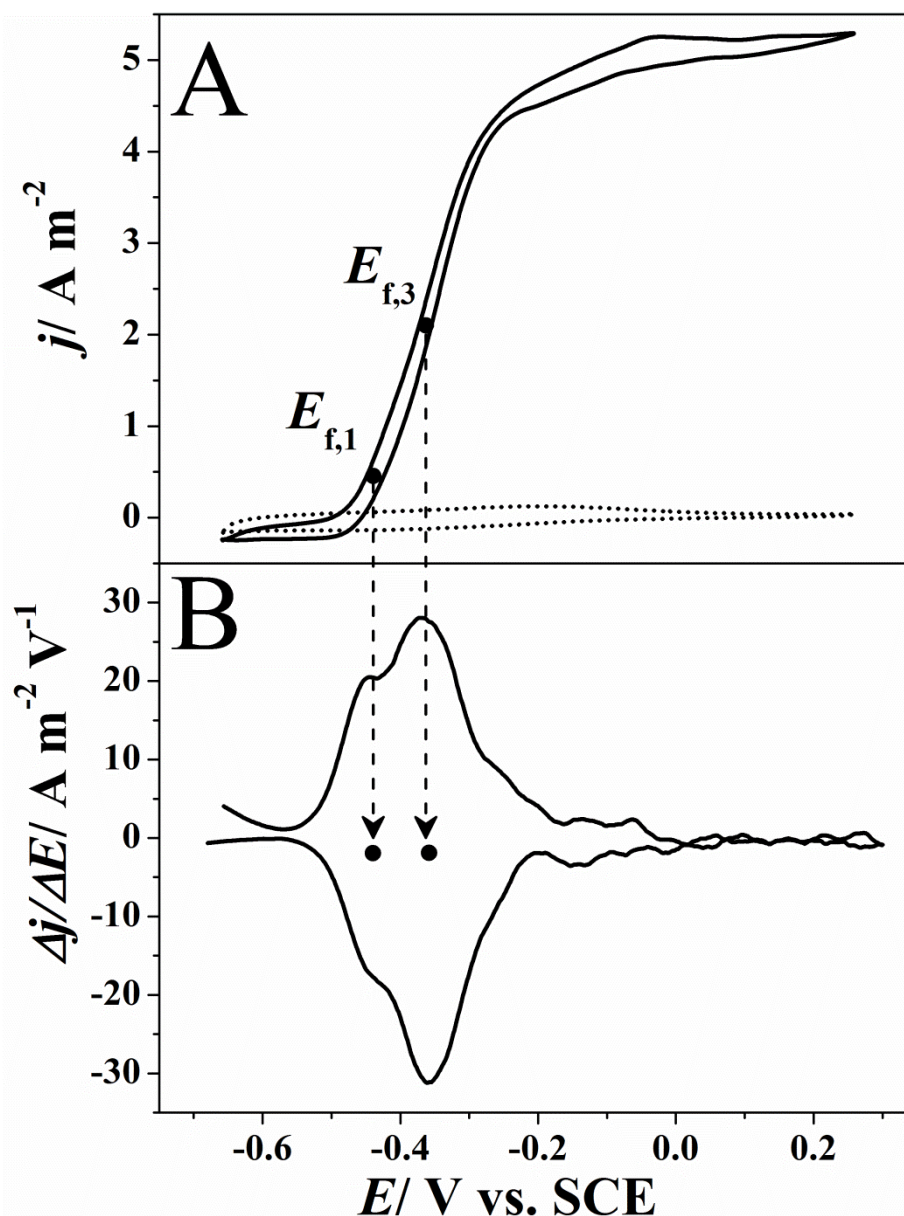
143 Under our experimental conditions, *Gb. sulfurreducens* was able to produce a higher j_{max}
144 (11.60 ± 2.21 A/m²) than the one obtained with *Glk. subterraneus* (see Table S1 and Fig. S1).
145 *Gb. sulfurreducens* biofilms obtained here produced results which corroborate previous

146 findings obtained by others with this model bacterium^{12, 28, 29, 51, 61}. This confirms that: 1) our
147 experimental methods are well standardized procedures comparable to the ones reported in
148 the current literature and that 2) *Gb. sulfurreducens* is the ARB producing the highest current
149 densities in a fresh water environment. In addition, a plausible reason why *Gb. sulfurreducens*
150 biofilms outperformed *Glk. subterraneus* in terms of j_{max} is related to the diversified ET
151 mechanism found in *Gb. sulfurreducens*. This is illustrated by the presence of several redox
152 couples in CV and higher biofilm electrode coverage as per CLSM (see below).

153 **2.2 Turnover CV analysis of *Geoalkalibacter subterraneus* in the presence of substrate**

154 To analyze the extracellular electron transfer mechanism occurring in *Glk. subterraneus*, CV
155 was performed during three different conditions as described in the Experimental section.
156 Representative CVs for both conditions are shown in Fig. 2 (turnover) and Fig. 3 (non-
157 turnover). As proof that the turnover and non-turnover CV signals are due to a biofilm formed
158 at the electrode surface, a control CV of the bare surface electrode immersed in growth
159 medium is included for comparison (dotted line in Fig. 2A and in Fig. 3A). The negative
160 control CV showed no appreciable signals.

161 Fig. 2A shows a representative turnover CV illustrating a sigmoidal shape typically found in
162 ARB able to produce high currents and thick biofilms like *Geobacter sulfurreducens* PCA¹²,
163 *Rhodospseudomonas palustris* DX-1⁴⁰, *Thermincola ferriacetica* DSMZ 14005²² and more
164 recently *Geoalkalibacter ferrihydriticus* DSM 17813³⁰. Badalamenti *et al.*³⁰ reported the CV
165 characterization of *Glk. subterraneus* biofilms but only under turnover conditions and
166 showing a sigmoidal shape. The present observations of turnover CV analysis corroborate the
167 findings of Badalamenti *et al.*³⁰. However, the existing differences are very likely due to
168 experimental procedures. When calculating the respective first derivative of the turnover CV
169 of *Glk. subterraneus*, under our experimental turnover conditions, two different inflection
170 points are revealed (Fig. 2B). A first inflection point with a formal potential ($E_{f,1}$) at $-446.5 \pm$
171 1.2 and a second clearly prominent inflection point $E_{f,3}$ at -364.8 ± 4.5 mV (vs. SCE), a
172 potential commonly ascribed to DET-proteins (see Fig S2). Likewise, multiple inflection
173 points in the first derivative (here defined as $E_{f,1}$) have been reported elsewhere for other
174 electroactive bacteria such as *Gb. sulfurreducens*^{27, 51, 55, 61} and *T. ferriacetica*⁴¹. Nevertheless,
175 it is not completely clear how these non prominent “additional” inflection points (such as $E_{f,1}$)
176 are involved in the overall ET mechanism⁵¹.



177

178 **Fig. 2** A) Representative turnover cyclic voltammogram of a *Geokalibacter subterraneus*
 179 biofilm (continuous line) and B) respective first derivative curve. As well in A), dotted line
 180 shows bare electrode CV performed before chronoamperometric biofilm growth.

181 When examining the turnover CV signal, it is clear that the CV shape shown here resembled
 182 the one seen for bacteria capable of performing a DET mechanism via outer membrane
 183 cytochromes (OMCs). Nevertheless, there is still no conclusive evidence whether OMCs are
 184 responsible for a DET mechanism in the electroactive biofilms of *Glk. subterraneus*.
 185 Moreover, the prominent inflection point potentials found here were very similar to the
 186 potential found for DET (see Fig. S2). As shown in Fig. S2, bacteria able to perform a DET
 187 mechanism presented an inflection point in a very broad potential window that ranged from -

188 500 to -300 mV vs. SCE. However these differences could be attributed to the particular
189 growth conditions (*e.g.*, pH, temperature, substrate, salt concentration, among others).

190 **2.2.1 Turnover CV comparison of *Geoalkalibacter subterraneus* and *Geobacter*** 191 ***sulfurreducens* biofilms**

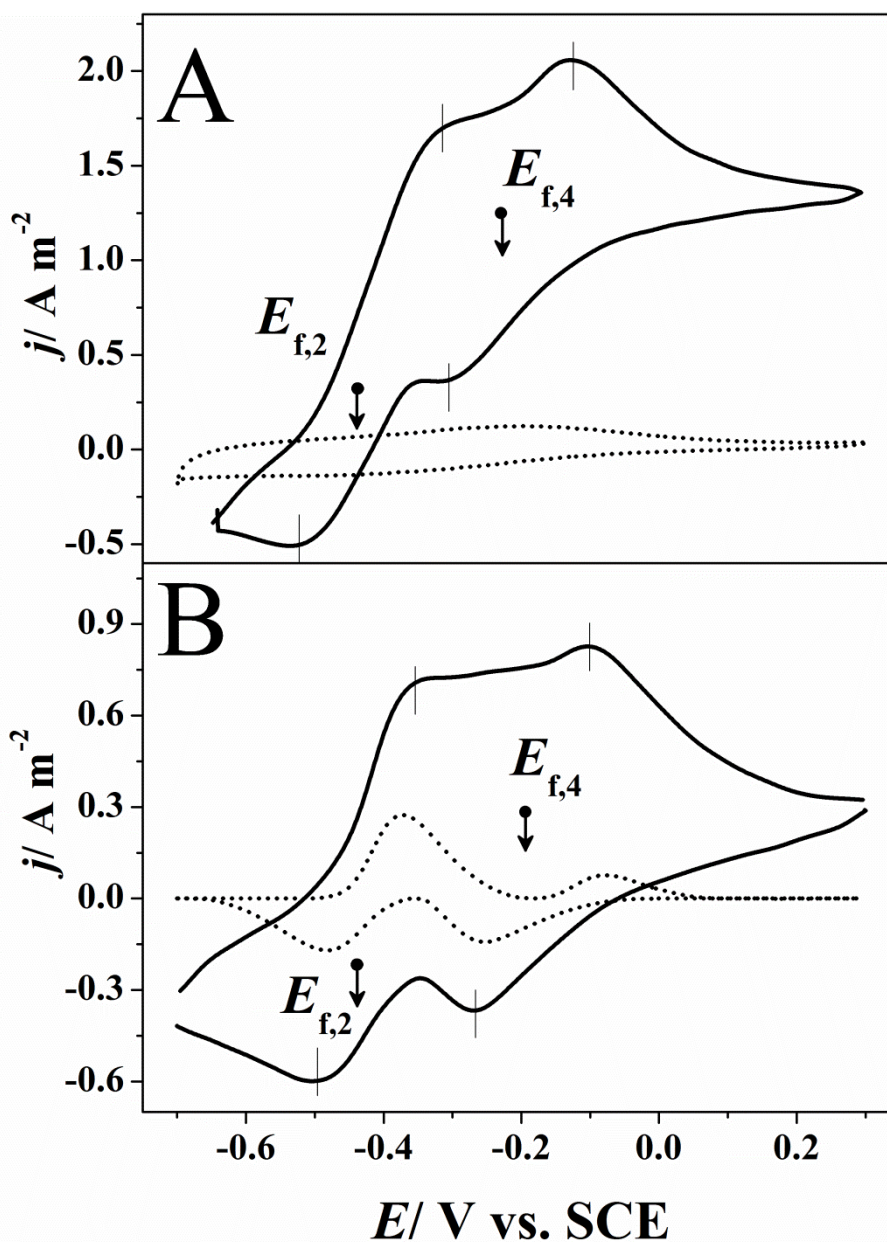
192 Further evidence of a DET mechanism performed by *Glk. subterraneus* was provided by a
193 comparative analysis conducted while performing electrochemical experiments to grow
194 electroactive biofilms of *Gb. sulfurreducens* (see Fig. S1). Fig. S3 shows an exemplary
195 turnover CV of a *Glk. subterraneus* biofilm compared to a turnover CV of a *Gb.*
196 *sulfurreducens* biofilm. As expected, *Gb. sulfurreducens* illustrated the typical sigmoidal
197 shape under turnover conditions obtained by others^{12, 27, 51, 55, 61} and its first derivative showed
198 as well two inflection points included in the potential window of $E_{f,1}$ and $E_{f,3}$ (see Fig. S3).
199 This provides additional information on the ability of *Glk. subterraneus* to perform a DET
200 mechanism and could indicate a similar ET process as in the case of *Gb. sulfurreducens*.

202 **2.3 Non-turnover CV analysis of *Geoalkalibacter subterraneus* in the absence of** 203 **substrate**

204 Fig. 3 shows exemplary non-turnover CVs of a *Glk. subterraneus* biofilm. Fig. 3A shows a
205 substrate-depleted CV when substrate was totally consumed (as per metabolite analysis)
206 during CA cycle and Fig. 3B shows a substrate-deprived CV when the substrate depleted
207 medium was replenished for fresh medium with no electron donor.

208 Both non-turnover CVs in Fig. 3A and B depict a very similar voltammogram shape.
209 Additionally, both CVs possess two apparent redox couples with formal potentials $E_{f,2}$ and
210 $E_{f,4}$. However, the substrate-depleted CV in Fig. 3A exhibits a catalytic behaviour with a
211 limiting current of about 2 A/m². When compared to Fig. 3B, the limiting current masks the
212 two redox couples that were clearly observed when CV was performed in total absence of
213 electron donor (under substrate deprived non-turnover CV). The catalytic behaviour observed
214 in Fig. 3A could be due to the presence of a substance (or substances) produced during CA.
215 Interestingly, the observed catalytic current of 2 A/m² during non-turnover CV in Fig. 3A was
216 in agreement with the current density observed at the end of the first CA cycle shown in Fig. 1
217 (residual current of about 1.3 A/m²).

218



219

220 **Fig. 3** A) Representative cyclic voltammogram of *Geokalibacter subterraneus* biofilm u
 221 substrate depleted conditions (continuous line) and cyclic voltammogram of bare electrode
 222 (dotted line); B) substrate deprived non-turnover cyclic voltammogram (dotted line provides
 223 the respective SOAS⁶² base-line subtracted curve).

224 Fig. 3B shows the SOAS⁶² base-line subtracted curve (dotted line) of the substrate deprived
 225 non-turnover CV (see Experimental section). The subtracted CV clearly depicts the position
 226 of two redox couples with formal potentials $E_{f,2}$ and $E_{f,4}$ at -427.6 ± 0.5 and -165.5 ± 2.6 mV
 227 vs. SCE, respectively. The value of $E_{f,2}$ was close to both formal potentials derived from first
 228 derivatives of CV under turnover conditions (see Fig. 2). This could indicate that the redox
 229 couple detected at $E_{f,2}$ was responsible for the bioelectrocatalytic electron transfer.

230 Nevertheless, the lack of an electrochemical characterization of the OMCs of *Glk.*
231 *subterraneus* prohibits an *a priori* assignment of $E_{f,2}$ to a certain type of protein (or multiple
232 proteins) responsible for the suggested DET mechanism.

233 **2.3.1 Non-turnover CV comparison of *Geoalkalibacter subterraneus* and *Geobacter*** 234 ***sulfurreducens* biofilms**

235 Further data supporting a putative DET by *Glk. subterraneus* comes from the comparison of
236 its non-turnover CV with the one obtained with *Gb. sulfurreducens* biofilms. Although non-
237 turnover CVs of *Glk. subterraneus* (with two redox systems) and *Gb. sulfurreducens* (with
238 four redox couples previously observed^{27, 51}) differed significantly (see Fig. S4), the value of
239 *Glk. subterraneus*'s formal potential $E_{f,2}$ was close to *Gb. sulfurreducens*'s $E_{f,Gb2}$. This
240 suggests a common DET mechanism in both bacteria. Based on this experimental data and on
241 previous studies of *Gb. sulfurreducens* that propose a DET via OMCs⁶³, we suggest to ascribe
242 the formal potential $E_{f,2}$ found here for *Glk. subterraneus* to a DET very likely via an OMC.
243 Future research should therefore concentrate on the investigation of the OMC (or OMCs)
244 involved in the DET mechanism of *Glk. subterraneus*.

245 The proper assignation of $E_{f,4}$ to a respective ET mechanism (Fig. 3B) was not a
246 straightforward task. Some authors previously suggested a possible explanation based on the
247 CV observed for *Gb. sulfurreducens* biofilms^{27, 51}. Here we propose to extrapolate such
248 explanation derived from the comparison of Fig. 2B and 3B to our results. From these figures
249 one can see that $E_{f,1}$, $E_{f,2}$ and $E_{f,3}$ share a very similar value. Therefore, it can be hypothesized
250 that these redox processes contributed to the bioelectrocatalytic anodic electron transfer. On
251 the other hand, since $E_{f,4}$ does not have a formal potential match in turnover conditions, it
252 appears to have no contribution in the overall ET mechanism.

253 **2.4 Imaging of *Geoalkalibacter subterraneus* biofilm surface coverage and thickness** 254 **using confocal laser scanning microscopy**

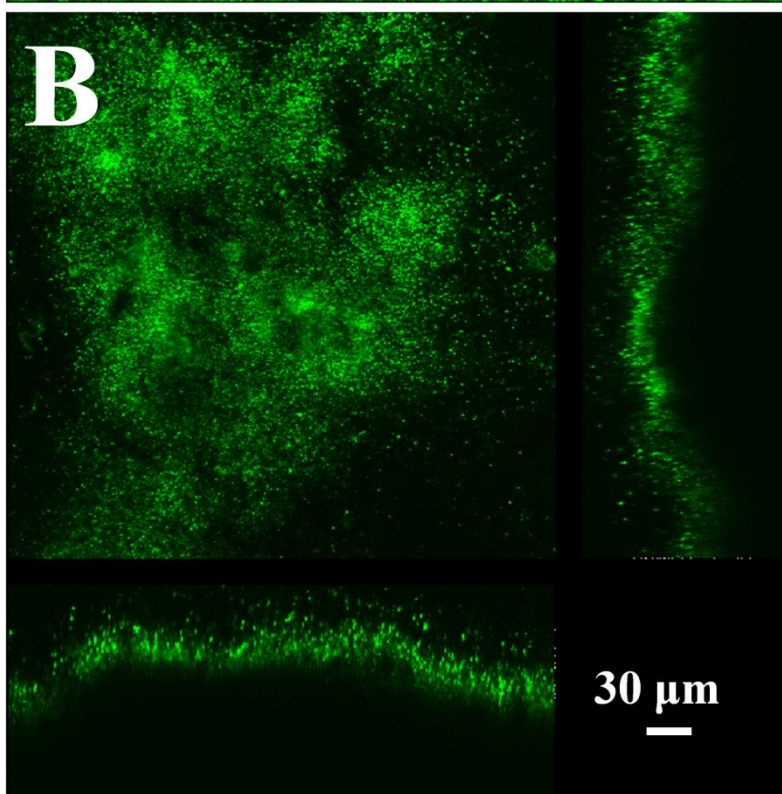
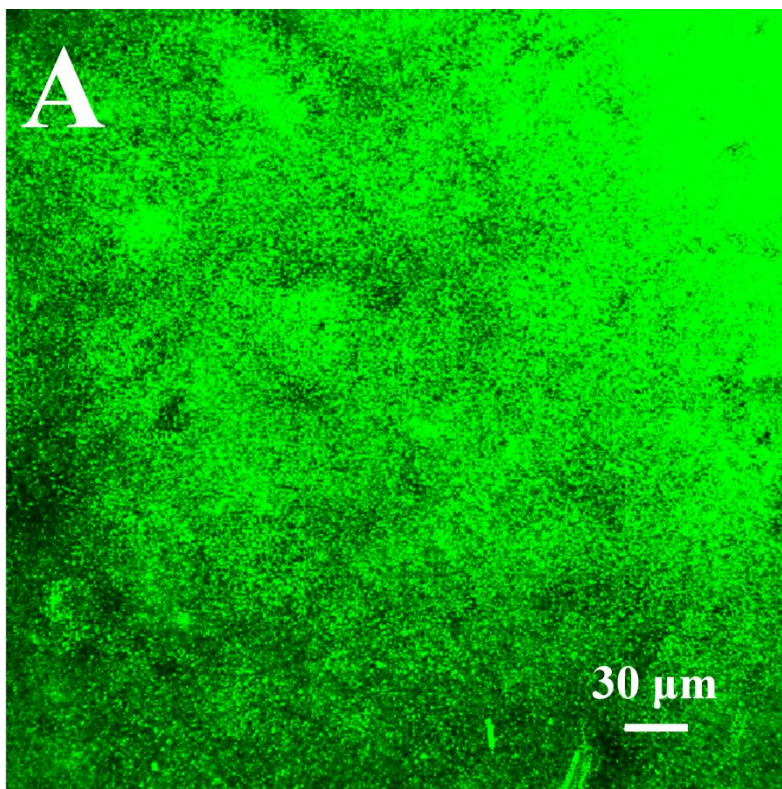
255 In order to study how *Glk. subterraneus* (and *Gb. sulfurreducens* for comparison) attached to
256 the electrode surface, confocal laser scanning microscopy (CLSM) was used to conduct a
257 qualitative (with LAS AF and Volocity®) description and a quantitative (with PHLIP) biofilm
258 analysis as described in the Experimental section. In this course, it has to be noticed that this
259 is the first CLSM description of *Glk. subterraneus* electroactive biofilms. CLSM was used
260 first of all, for its advantage over Scanning electron microscopy for quantitatively analyzing
261 in depth the layers across the biofilm and hence, allowing the calculation of surface coverage
262 and thickness⁶⁴. Second, CLSM allows a live biofilm to be imaged by avoiding a harsh drying

263 procedure that could change its morphology. In our experiments the biofilms were stained
264 with a LIVE/DEAD® kit. Bacterial cells embed in biofilms stained green which could
265 indicate that most of the cells were still metabolically active at the moment of the CLSM
266 analysis¹⁷. However, since this is the first work reporting the use of this kit with biofilms of
267 *Glk. subterraneus*, the successful staining of dead cells of this bacterium should be still
268 experimentally tested. The maximum intensity projection of a *Glk. subterraneus* biofilm is
269 shown in Fig. 4A. This picture confirms a very uniform coverage of the electrode surface as
270 previously observed by visual inspection of a reddish biofilm (see Fig. S5). Additionally,
271 when analyzing the different slices that compose the maximum intensity projection, it is clear
272 that the biofilm was constituted of a stack of several biofilm monolayers (see Fig. S6). From
273 the different orthogonal cross sections made throughout the biofilm, it seems that the biofilm
274 is about 30 µm thick (Fig. 4B). However, this idea was later discarded by PHLIP quantitative
275 analysis which showed a biofilm with a thickness value of 76 ± 7 µm as described in the
276 following section. Further 3D reconstruction with Volocity® revealed a very peculiar biofilm
277 structure (see Fig. S7) composed of an undulating structure distributed across the analyzed
278 electrode surface sample probably caused by biofilm overgrown which matches previous
279 observations in *Gb. sulfurreducens* biofilms^{17, 51}.

280 **2.4.1 CLSM comparison of *Geoalkalibacter subterraneus* and *Geobacter sulfurreducens*** 281 **biofilms**

282 The comparison of *Glk. subterraneus* and *Gb. sulfurreducens* CLSM pictures is shown in Fig.
283 S8 and in Table S3. It was observed that *Glk. subterraneus* produced a lower current density
284 (4.68 ± 0.54 A/m²) when it formed a thicker biofilm (76 ± 7 µm) than *Gb. sulfurreducens*.
285 Nevertheless, the average electrode coverage value of *Gb. sulfurreducens* (31 ± 16 %) is
286 higher than the one of *Glk. subterraneus* (23 ± 7 %) and therefore indicating that these
287 differences in biofilm architecture contribute to explain the higher current density by *Gb.*
288 *sulfurreducens*. A similar trend was recently reported by Richter *et al.*⁶⁵. They studied *Gb.*
289 *sulfurreducens* wild-type and the mutant *pilA4*, a strain expressing only the short isoform of
290 the PilA preprotein that composes the type IV pili of *Gb. sulfurreducens*, a protein essential
291 for DET. Therefore, diminishing the ability of the mutant to effectively transfer electrons to
292 insoluble Fe(III) oxides and graphite anodes. Whereas the wild-type of *Gb. sulfurreducens*
293 produced a higher current density, it showed a thinner biofilm but higher electrode coverage
294 value than the mutant.

295 One issue that emerges from the thickness observed in *Glk. subterraneus* is that the value is
296 very high for the values normally reported in the literature for pure culture electroactive
297 bacteria¹⁴⁻¹⁸. Thus, to be the first CLSM description of this strain, it is advisable and
298 justifiable to further investigate the possible reasons behind the biofilms produced by this
299 electroactive bacterial species.



300

301 **Fig. 4** Exemplary confocal laser scanning microscopy of *Geoalkalibacter subterraneus*
302 biofilms grown on graphite planar electrodes. A) Maximum intensity projection and B)
303 Orthogonal cross section of a single slice through the biofilm with top and right panels
304 representing perpendicular slices.

305 **2.4.2 Proof that the presence of an electroactive biofilm is due to metabolically active** 306 **bacterial cells transferring electrons to the electrode**

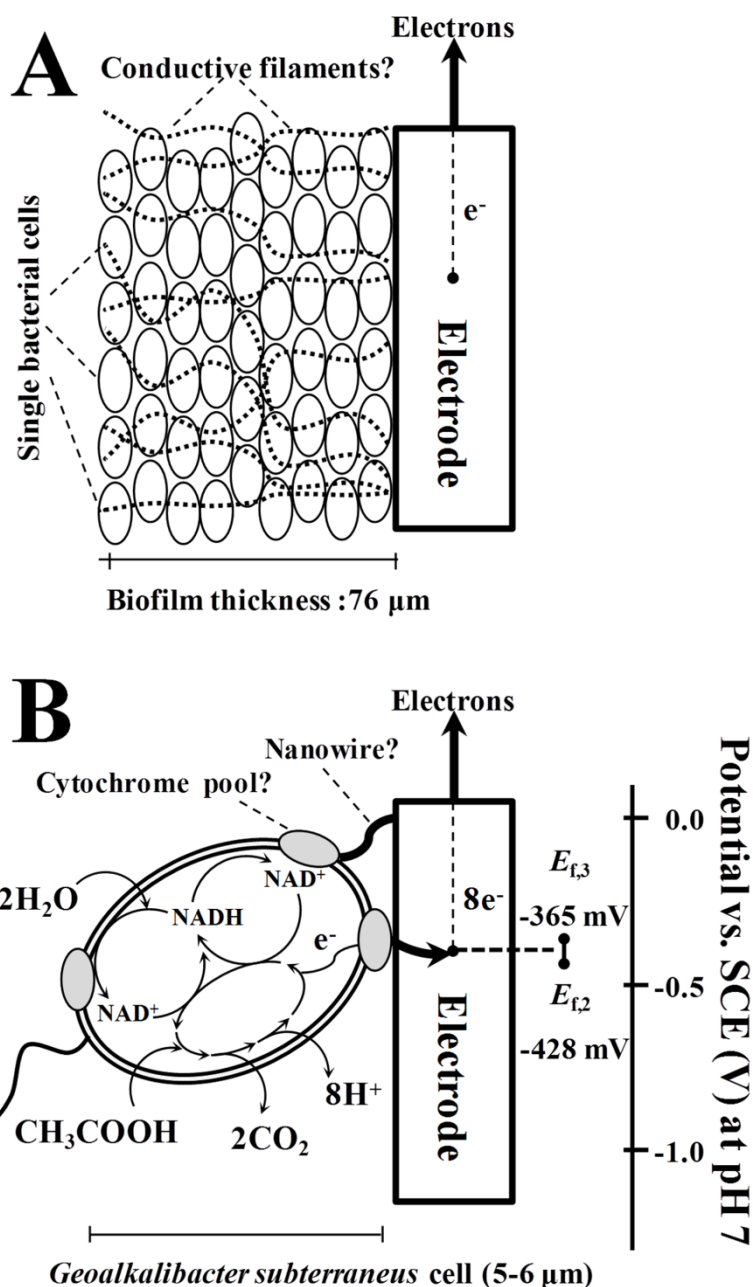
307 To support that the biofilm formation was due to the electrons harvested from the substrate
308 oxidation followed by a later release to the electrode surface by the electroactive biofilm,
309 CLSM images were compared to a non-electrochemical negative control. This control was an
310 electrode placed in the same electrochemical cell of a *Glk. subterraneus* electrochemical
311 experiment but not potentiostatically controlled (*i.e.*, the electrode was immersed in the same
312 growth medium during the electrochemical biofilm growth). The result of such CLSM
313 observations is shown in Fig. S8 and Table S3. When comparing CLSM pictures in Fig. S8,
314 the difference between both maximum intensity projections and orthogonal cross sections is
315 very clear. While for the potentiostatically controlled electrode there was an evident thick
316 biofilm formed of metabolically active bacteria, for the negative control no significant
317 coverage of the electrode surface and biofilm formation was observed (see Table S3). These
318 findings are consistent with the observations made by Malvankar *et al.* (2011)⁶⁶. They utilized
319 in a similar way a non-connected electrode as negative control electrode. The values of
320 electrode coverage and thickness calculated here for the negative control electrode suggest
321 that the bacterial cells stained with the LIVE/DEAD kit corresponded to a heterogenous
322 deposition of bacterial cells likely de-attached from the potentiostatically controlled electrode
323 in which *Glk. subterraneus* was forming an electroactive biofilm (Fig. S8).

324 These CLSM findings are well in line with previous studies of ARB able to produce high
325 currents and thick biofilms such as *Geobacter sulfurreducens*^{15, 17, 29, 51, 66} and *Thermincola*
326 *ferriacetica*²² but in clear contrast to other low current producing bacteria like *Shewanella*
327 *species*¹⁹. On the other side, similar observations of biofilm coverage of an electrode material
328 were obtained with Scanning electron microscopy measurements in *Rhodospseudomonas*
329 *palustris* DX-1⁴⁰, *Geoalkalibacter ferrihydriticus*³⁰ and *Geolkalibacter subterraneus*³⁰.

330

331 **3 Conclusions**

332 The present study on the characterization of *Geoalkalibacter subterraneus* is summarized in
333 Fig. 5 and indicates the following findings: CA exhibited the highest current density ($4.68 \pm$
334 0.54 A/m^2) produced on a planar electrode for a pure culture of an ARB under saline
335 conditions (3.5% NaCl). CV under turnover and non-turnover conditions made evident the
336 appearance of two different redox couples for each condition. When comparing their formal
337 potentials, two of them had a very close value ($E_{f,2}$ and $E_{f,3}$) indicating that the molecule (or
338 molecules) responsible for the ET mechanism might fall between the potential window
339 delimited by $E_{f,2}$ and $E_{f,3}$. CLSM confirmed a biofilm composed of several layers of
340 metabolically active bacteria that spread all over the electrode material with a thick biofilm up
341 to $76 \pm 7 \mu\text{m}$ height but a low electrode coverage of only $23 \pm 7 \%$. From these findings, it is
342 then proposed that *Geoalkalibacter subterraneus* performs a DET mechanism in contact with
343 the electrode material. Nevertheless, to firmly determine the molecule responsible for the ET
344 mechanism future work will be performed by employing Surface Enhanced Resonance
345 Raman Spectroscopy, a technique successfully used to detect selectively cellular membrane
346 redox proteins in proximity of the electrode surface^{63, 67, 68}.



347

348 **Fig. 5** Conceptual illustration of the DET mechanism performed by *Geobacter*

349 *subterraneus* derived from the electrochemical and microscopic characterization

350 accomplished here. A) Representation of the visible apparent biofilm with a 70-80 μm

351 thickness that could possibly contain conductive filaments³⁰ to transport electrons by the so

352 called long-range ET⁶⁹ through the biofilm composed of monolayers of stained green cells as

353 per CLSM analysis; B) Schematic proposal at the single cell level²⁶ accomplishing the

354 oxidation of acetate to produce carbon dioxide and harvest of electrons, for instance, by the

355 tricarboxylic acid cycle⁷⁰ to perform a DET mechanism via a putative cytochrome (or

356 cytochrome pool) with a formal potential “conduit” (E_f) between -428 and -365 mV vs. SCE

357 determined during CV analysis.

358 **4 Experimental**

359 **4.1 General conditions**

360 All chemicals were of analytical or biochemical grade and were purchased from Sigma-
361 Aldrich and Merck. If not stated otherwise, all potentials provided in this manuscript refer to
362 the SCE reference electrode (KCl 3.0 M, +240 mV vs. SHE, Materials Mates, La Guilletière
363 38700 Sarcenas, France). All media preparations were adjusted to pH 7, vigorously flushed
364 with N₂ gas (purity ≥ 99.9999, Linde France S.A.) for at least 30 min using a commercial air
365 stone (or aquarium bubbler) and then autoclaved (121°C for 20 min). Bioelectrochemical
366 experiments were conducted under potentiostatic control and strictly anoxic^{71, 72} and sterile
367 conditions. All incubations were performed at 37°C.

368 **4.2 Metabolite and biogas analysis**

369 Acetate consumption was determined by liquid injection into a gas chromatograph (GC 8000,
370 Fisons Instruments) according to Aceves-Lara *et al.*, 2008⁷³. Biogas composition (CH₄, CO₂,
371 H₂ and N₂) was determined using a gas chromatograph (Clarus 580, Perkin Elmer) coupled to
372 Thermal Catharometric detection, as described elsewhere⁷⁴.

373 **4.3 Cell cultures and media**

374 *Geoalkalibacter subterraneus* strain Red1 was purchased from DSMZ (DSM No.: 23483,
375 German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany).
376 Sterile growth medium FRR²⁶ was used for routinely culture maintenance and contained (per
377 L): 17.0 g of NaCl, 4.50 g of MgCl₂·6H₂O, 0.35 g of CaCl₂·2H₂O, 1.00 g of NH₄Cl, 0.08 g
378 KH₂PO₄, 3.50 g of NaHCO₃, 3.00 g of Yeast extract, 1 mL of trace element solution, 1 mL of
379 selenite-tungstate solution, 2.55 g of NaNO₃ as final electron acceptor when *Glk.*
380 *subterraneus* cells were harvested for further growth in electrochemical cells (as described
381 below) and 1.00 g of CH₃COONa as electron donor.

382 As stated before, the novel ARB *Glk. subterraneus* was the bacterium dominating high
383 current producing biofilms in previous studies of our group³¹. Therefore, for the growth of
384 *Glk. subterraneus* biofilms on graphite electrodes (*i.e.*, in electrochemical experiments) two
385 similar media compositions were used. Medium FRR²⁶ without NaNO₃ and modified Starkey
386 medium as reported elsewhere⁷⁵ were used. The later was used previously for the selection
387 and identification of *Glk. subterraneus* in a potentiostically controlled BES³¹. Starkey medium
388 contained per liter: 35.0 g NaCl, 0.5 g K₂HPO₄, 2.0 g NH₄Cl, 7.6 g MES buffer, 0.2 g Yeast
389 Extract, 1 mL of oligo-elements solution and 10 mM sodium acetate as electron donor. Oligo-
390 elements solution contained (per L): 46 mL HCl 37%, 55 g MgCl₂·6H₂O, 7.0 g

391 FeSO₄(NH₄)₂SO₄·6H₂O, 1.0 g ZnCl₂·2H₂O, 1.2 g MnCl₂·4H₂O, 0.4 g CuSO₄·5H₂O, 1.3 g
392 CoSO₄·7H₂O, 0.1 g BO₃H₃, 1.0 g Mo₇O₂₄(NH₄)₆·4H₂O, 0.05 g NiCl₂·6H₂O, 0.01 g
393 Na₂SeO₃·5H₂O and 60.0 g CaCl₂·2H₂O. The examination of the two media in electrochemical
394 experiments inoculated with *Glk. subterraneus* led to similar performance (see Fig.S1 and
395 Table S2).

396 As stated before, to conduct an accurate comparison of the performance obtained with *Glk.*
397 *subterraneus*, parallel electrochemical experiments were carried out with *Gb. sulfurreducens*
398 (DSM strain number 12127). *Gb. sulfurreducens* cells were harvested for electrochemical
399 experiments as reported elsewhere^{28, 60, 76}, except during growth on graphite electrodes. In
400 experiments reported here with *Gb. sulfurreducens*, no gas was flushed during the growth of
401 biofilms in electrochemical experiments.

402 **4.4 Electrode preparation**

403 Preparation of electrodes was according to the procedure reported elsewhere⁷⁷. In brief:
404 working electrodes were 2.5 cm x 2.5 cm x 0.25 cm (total immersed projected electrode
405 surface area of 15 cm²) planar graphite plates (C000440/15, Goodfellow SARL, 229 Rue
406 Solférino, F-59000 Lille, France) screwed onto 2 mm diameter, 15 cm long titanium rods
407 (TI007910/13, Goodfellow) that ensured electrical connection. Planar graphite electrodes
408 were used as delivered by the provider. Counter electrodes were 90% Platinum-10% Iridium
409 grids joint by heating in a blue flame with a 0.5 mm diameter, 15 cm long 90% Platinum-10%
410 Iridium rod (Heraeus PSP S.A.S., Contact Materials Division, 526, Route des Gorges du
411 Sierroz, 73100 Grésy-sur-Aix France).

412 **4.5 Bioelectrochemical set-up and experiments**

413 *Glk. subterraneus* and *Gb. sulfurreducens* biofilms were grown in potentiostatically
414 controlled half-cells (autoclavable borosilicate glass) containing 500 mL of solution with
415 around 200 mL of headspace as reported elsewhere⁷⁷. The lid and the reactor body were
416 sealed with a clamping ring. Biofilm growth was performed in semi-batch
417 chronoamperometric (CA) experiments with media replacement as described previously¹⁹ at a
418 fixed applied potential of +200 mV using a Potentiostat/Galvanostat VMP3 (BioLogic
419 Science Instruments, France). After half-cells containing electrodes were completely
420 assembled (*i.e.*, before inoculation of bacterial cells) anoxic conditions were assured similarly
421 as in previous reports^{28, 60, 76}. Here, we flushed the media with high purity N₂ (≥ 99.9999 %)
422 for at least 30 min. The final composition of the gas phase was typically a mixture of O₂: 1.41
423 ± 1.16 % and N₂: 98.56 ± 1.18 %.

424 Inoculation of bacterial cells was carried out as previously described¹⁹. Concisely, 50 mL of
425 media in the early stationary phase (i.e., OD⁶²⁰ approx. 0.3-0.4) were anoxically sampled^{71, 72}
426 and centrifuged at 3000 rpm for 10 min. The pellet was re-suspended in 10 mL of the
427 respective media for electrochemical experiments and injected in the electrochemical cell (see
428 Fig. S9).

429 **4.6 Cyclic voltammetry (CV) for an insight into the electron transfer (ET) mechanism**

430 Different types of CVs were recorded during experiments according to Refs.^{19, 27, 78} (see Fig.
431 1). Control CV of the bare graphite electrode immersed in growth media before starting CA
432 and inoculating the half-cell (dotted line in Fig. 2A), turnover CV of a *Glk. subterraneus*
433 biofilm, i.e. at bioelectrocatalytic substrate consumption (continuous line in Fig. 2A) and two
434 types of non-turnover CV of a *Glk. subterraneus* biofilm: during substrate depleted conditions
435 (continuous line in Fig. 3A) and during substrate deprived conditions (continuous line in Fig.
436 3B).

437 **4.7 Data processing**

438 CA maximum current densities (j_{max}) of established microbial biofilms were analyzed
439 considering the total immersed electrode surface area since electroactive biofilms covered
440 both sides of the working electrode as per our observations (i.e., not only the side of graphite
441 working electrode facing Pt-Ir counter electrode, see Fig. S5). Here all data are based on
442 experiments of at least two independent biofilm replicates⁷⁹ and standard deviations are
443 presented through all the manuscript. For in-depth data analyses of CV, the open-source
444 software SOAS⁶² was used for baseline (capacitive current) correction for non-turnover
445 conditions.

446 **4.8 Confocal laser scanning microscopy (CLSM) to measure biofilm electrode coverage** 447 **and thickness**

448 *Glk. subterraneus* biofilms (and *Gb. sulfurreducens* for comparison) grown on planar graphite
449 plate electrodes were examined by CLSM after staining with nucleic acid-specific
450 fluorochromes. For this purpose, whole electrodes were mounted on a plastic petri dish and
451 subsequently stained with the LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit (Invitrogen)
452 as proposed by the manufacturer. Stained biofilms were covered with tap water and confocal
453 images of electroactive biofilms were acquired with a confocal laser scanning system (Leica
454 TCS SP2, Leica Microsystems, Wetzlar, Germany) using a 40x water immersion objective
455 (numerical aperture 0.8). Biofilms were observed with the 488 nm ray line of an argon laser
456 for excitation and the emitted light was collected in the 495- 616 nm spectral range. The

457 system was controlled by the Leica LCS software (version 2.61) (Leica, Germany). Each
458 electroactive biofilm attached to the electrode was scanned for CLSM images at three
459 different random locations; therefore, CLSM images were representative of the entire
460 electrode. These images were qualitatively inspected (LAS AF 2.4.1 build 6384, Leica
461 Microsystems and Volocity[®] Demo Version 6.2.1⁸⁰, PerkinElmer). Images were later
462 thresholded at 60⁸¹ and quantitatively analyzed with PHobia Laser scanning microscopy
463 Imaging Processor 0.3 (PHLIP)⁸², an open source public license Matlab toolbox commonly
464 used for the analysis of electroactive biofilms^{15, 65, 66}.

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471

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