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2 **Analysis of electron transfer dynamics in mixed**
3 **community electroactive microbial biofilms**

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17 microscopy, electron transfer, cytochromes, biofilms.

18

19 **Abstract**

20 Mixed community electroactive organisms form multi-layered biofilms that are
21 able to produce current densities comparable to those of pure *Geobacter*
22 *sulfurreducens*, an extensively studied metal-reducing organism. The
23 long-range electron transfer (ET) inside the biofilms and at the
24 biofilm/electrode interface was proven to be promoted by a network of outer
25 membrane cytochromes (OMCs). In the present work, we investigate the
26 electron transfer process in mixed community biofilms grown on Indium Tin
27 Oxide (ITO) electrodes by combining electrochemical measurements with
28 Confocal Resonance Raman Microscopy (CRRM) under potentiostatic control
29 and during chronoamperometry (CA). This approach allowed direct
30 comparison of the heterogeneous redox process at the biofilm/electrode
31 interface with the long-range OMCs-mediated ET inside the bulk biofilm. Our
32 work shows that: (i) during substrate oxidation, all OMCs are in the reduced
33 state at any distance from the electrode, and no concentration gradient of
34 oxidized OMCs is observed; (ii) the rate constant for the long-range,
35 homogeneous ET (k_{hom}^0) is 0.028 s^{-1} , which is considerably lower than that
36 predicted by others under the hypothesis that homogeneous ET is promoted
37 by OMCs alone, and may thus indicate the contribution of alternative fast
38 electron transfer processes; (iii) the metabolic respiration rate is much faster
39 compared to both homogeneous and heterogeneous ET, which have similar
40 rate constants. All in all, our results suggest that differences exist in electron
41 transfer mechanisms between mixed community and *G. sulfurreducens*
42 electroactive biofilms.

43 **Introduction**

44 Investigation of electron transfer (ET) mechanisms in electroactive microbial
45 biofilms (that is, biofilm capable of extracellular electron communication with
46 electrodes) has motivated substantial research efforts in recent years.
47 Unraveling electron conduction in microbes is relevant not only to engineered
48 devices such as biosensors and bioelectrochemical systems, but it can also

49 help explaining important microbial physiological processes governing natural
50 geochemical cycles in sediments. Amongst the most studied microbial players
51 in electroactive biofilms are bacteria belonging to the genus *Geobacter* and
52 *Shewanella*, because of the high current densities achievable at electrodes,
53 and their importance as model organisms of ET modes, respectively.
54 *Geobacter sulfurreducens* can develop thick biofilms on electrode surfaces,
55 spanning distances exceeding 100 μm . The mechanisms by means of which
56 electrons are transported through such long distances is the subject of
57 significant debate.¹⁻³ Proposed models include a) redox conduction, that is,
58 electron transfer through incoherent multistep electron hopping between
59 discrete redox cofactors bound to the biofilms,⁴ b) metallic-like conductivity,
60 according to which electron transfer occurs through intrinsic conducting
61 properties of extracellular appendages (called nanowires) specific to
62 *Geobacter sulfurreducens*.⁵ Redox conduction is based on the relatively high
63 abundance of multiheme *c*-type cytochromes located on the outer membrane
64 and periplasmic space, as well as along extracellular filaments, and dispersed
65 in the extracellular polymeric substance.⁶ On the contrary, metallic-like
66 conduction excludes hopping between cytochromes,^{7,8} since the inter-heme
67 spacing - measured by immune-gold labelling outside of the
68 bioelectrochemical system - is too large for electron hopping to occur.⁹ While
69 sufficient structural information regarding the spatial organization of heme-
70 proteins in biofilms under physiologically relevant conditions is still missing,
71 independent electrochemical and spectroscopy measurements seems to
72 agree on the important role played by *c*-type cytochromes in wiring the
73 electroactive biofilms to electrodes and providing electric conductivity.^{1,10-21}
74 Redox conduction is described by a detailed model consisting of a fast
75 heterogeneous electron transfer at the biofilm/electrode interface governed by
76 electron tunnelling, coupled with a slower long-range homogeneous electron
77 transfer occurring via a hopping mechanism.^{17,18} This model requires a redox
78 gradient as driving force to allow ET to a given direction.²² Recently, Bonanni
79 *et al.*²³ proposed a model where the contribution of both OMCs and nanowires
80 are considered. While this seems to explain electron conduction in *G.*
81 *sulfurreducens* biofilms, electron transfer may be different in mixed culture

82 films, where the presence of multiple species may add complexity to the
83 already complicated spectrum of synergies and competitions that are
84 expected to arise for available substrates and the terminal electron acceptor
85 (*i.e.*, the anode). While studying conduction in model organisms such as *G.*
86 *sulfurreducens* is important from a physiological and mechanistic point of
87 view, understanding ET in broader mixed culture microbial aggregates is
88 essential also because of the role they play in existing and potential
89 applications in environmental and industrial biotechnologies.²⁴⁻²⁶

90 Spectroelectrochemical methods that derive from the combination of
91 electrochemical techniques with various spectroscopies provide great
92 opportunities for the study of electron conduction in these systems. Compared
93 to established electrochemical methods such as cyclic voltammetry (CV),
94 techniques based on Raman scattering provide important structural
95 information of the matrix based on spectral fingerprint of key molecules.²⁷
96 Resonance Raman (RR) spectroscopy is particularly suitable for the study of
97 electron transfer involving cytochromes because of the signal enhancement
98 achieved when the frequency of the excitation laser line is in proximity to an
99 electronic transition of the heme group in a cytochrome. This selectivity
100 towards heme-containing proteins together with the spatial resolution
101 achievable by combining RR spectroscopy with microscopy (yielding confocal
102 resonance Raman microscopy, CRRM), allows the use of CRRM to monitor
103 surface-exposed cytochromes promoting the long-range ET at any distance
104 from the electrode surface, as well as periplasmic cytochromes involved in
105 cells metabolism and extracellular ET. However, due to their abundance and
106 accessibility, surface-exposed proteins, hereby denoted as OMCs, dominate
107 the spectrum.¹³ Using this approach, we have previously resolved the spatial
108 distribution²⁸ and redox state²⁰ of OMCs of biofilms *in vivo*, without impacting
109 on the catalytic activity of mixed culture biofilms.

110 Herein, we improved our experimental set up and devised a miniature
111 spectroelectrochemical cell (schematized in Figure S1) equipped with
112 transparent Indium Tin Oxide (ITO) working electrodes specifically designed
113 to allow simultaneous electrochemical and CRRM measurements on the
114 biofilms directly (that is, *in situ*) without the need to transfer the
115 biofilm/electrodes from the electrochemical cell to the microscope stage; a

116 procedure that inevitably exposes the biofilms to air, albeit briefly. This new
117 set up allows to compare our results on mixed cultures to what others have
118 done on *G. sulfurreducens* biofilms.¹⁶ This approach excludes that differences
119 observed for mixed cultures and *G. sulfurreducens* are due to the
120 experimental configuration such as the focusing of the laser beam and the
121 electrode material. Moreover, CRRM hereby applied in the time-resolved
122 mode, was used to support the interpretation of simultaneous
123 chronoamperometry (CA) and CRRM measurements. To our knowledge, this
124 combined approach was never attempted before, and it turned out to provide
125 much direct information about the rate limiting steps of electron transfer
126 processes in electroactive microbial biofilms.

127 **Material and Methods**

128 **Biofilms formation and growth medium**

129 Primary biofilms were enriched using domestic wastewater from a local wet
130 well as the inoculum and incubated in a sealed single-chambered
131 bioelectrochemical system consisting of two carbon rods (Morgan AM&T,
132 Australia) serving as working and counter electrodes, and a Ag/AgCl
133 reference electrode in 3 M KCl (MF-2052, Basi, USA). All potentials herein are
134 reported with respect to this reference electrode (+0.210 V vs the standard
135 hydrogen electrode, SHE). The electrodes were immersed into 400 mL sterile
136 anaerobic media (composition below) purged with pure nitrogen for at least 30
137 minutes to ensure anoxic conditions. The working electrode was poised at 0 V
138 using a potentiostat (Potentiostat/Galvanostat VMP3, BioLogic Science
139 Instruments, France). Biofilm growth was monitored by measurement of
140 bioelectrocatalytic current production and by regular cyclic voltammetry
141 analysis. The media was regularly exchanged (usually once per week).
142 Primary biofilms were scraped off the rods using a sterile blade and used as
143 inoculum for the formation of secondary biofilms that were used in this study.
144 Both primary and secondary biofilms were grown at room temperature
145 (22±1)°C. The growth medium consisted of autoclaved deionized water
146 containing: Na₂HPO₄ (6.0 g L⁻¹), KH₂PO₄ (3.0 g L⁻¹), NH₄Cl (0.1 g L⁻¹), NaCl
147 (0.5 g L⁻¹), MgSO₄·7H₂O (0.1 g L⁻¹), CaCl₂·2H₂O (0.015 g L⁻¹), trace

148 elements solution (1 mL L^{-1} , composition in Lu *et al.*²⁹). Sodium acetate
149 (CH_3COONa) was used as metabolic substrate at concentrations that ranged
150 between 1 and 20 mM as indicated in the text.

151 **Spectroelectrochemical cells**

152 The spectroelectrochemical cells used for CRRM measurements were
153 designed to allow for observations of electroactive biofilms *in vivo* and *in situ*.
154 A schematic cell is provided in Figure S1 in the Supplementary Information
155 (SI). It consists of single chambered miniature BESs obtained by etching a 10
156 x 10 mm well into a 10 mm thick polycarbonate frame. Small channels were
157 drilled on the sides of the frame to accommodate the reference electrode and
158 the counter electrode (consisting of a platinum wire). The working electrode
159 consisted of 20 x 20 mm sodalime glass cover slip (thickness 0.5 mm) coated
160 with 100 nm of Indium Tin Oxide (ITO 90/10 wt%, 99.99%, Testbourne Ltd.,
161 UK) deposited using an electron beam evaporator (BJD2000, Temescal,
162 USA). The use of ITO permitted to perform confocal analysis directly from the
163 outside of the electrochemical cells, without the need to remove the biofilm
164 from the medium and expose it to air, albeit for a brief period of time. The
165 glass/ITO sandwich was plasma-treated to render the surface highly
166 hydrophilic and favour bacterial attachment,³⁰ and it was then glued onto one
167 side of the well using silicone glue, resulting in an effective (exposed) area of
168 the electrode of 1 cm^2 . Finally, a glass cover slip was glued to the opposite
169 side of the well to close the electrochemical chamber, resulting in an internal
170 volume of 1 mL. The electrochemical cells were fed using a multichannel
171 syringe pump (NE-1600, New Era Pump Systems, USA) at the flow rate
172 ranging from 0.1 to of 1 mL h^{-1} . External connection of the ITO was obtained
173 by gluing titanium wires on the portion of the ITO layer outside of the chamber
174 using conductive epoxy glue (MG chemicals, USA). Working, counter, and
175 reference electrodes were typically connected to a multichannel potentiostat
176 (CHI1000B, CH Instruments, USA). For electrochemical measurements
177 during Raman spectra acquisition, a VMP3 Potentiostat/Galvanostat (BioLogic
178 Science Instruments, France) was used. Biofilms were grown at a potential of
179 0 V. Measurements of catalytic current production over time and of cyclic

180 voltammograms were used to monitor the electrochemical activity of the
181 biofilms.

182 **Confocal Resonance Raman Microscopy measurements**

183 All CRRM measurements were performed at room temperature ($22\pm 1^\circ\text{C}$)
184 using an Alpha 300 Raman/AFM (WITec GmbH, Ulm, Germany) equipped
185 with a frequency-doubled continuous-wave Nd:YAG laser to obtain a 532 nm
186 excitation line. The laser beam was focused by an objective lens (Nikon 40X,
187 N.A. 0.6, CFI S Plan Fluor ELWD objective). The back-scattered Raman light
188 from the sample was collected with a 100 μm optical fibre employing a Raman
189 spectrometer (1800 grooves per mm grating) with a charge-coupled device
190 (EMCCD) spectroscopic detector. Biofilm stability upon exposure to the 532
191 nm laser was tested by measuring changes of the signal intensity of the
192 vibrational mode ν_{15} (sensitive to *c*-type heme groups, *vide infra*) at 750 cm^{-1}
193 during a 300 s continuous exposure test (Figure S2). Measurements
194 confirmed that changes of signal intensity for the mode ν_{15} were negligible
195 ($<2.4\text{ CCD counts s}^{-1}$) for laser powers less than $430\text{ }\mu\text{W}$ (measured at the
196 sample using a power meter (Thor Labs, USA)). Project FOUR software
197 (WITec GmbH, Ulm, Germany) was used for spectra processing and image
198 reconstruction. OriginPro 9.1 software (OriginLab, Northampton, USA) was
199 used for data fitting. Depth measurements on biofilm sections were done by
200 collecting individual spectra over a total of 12 points spaced $5\text{ }\mu\text{m}$ from each
201 other each (covering $60\text{ }\mu\text{m}$ -thick sections), and using an integration time of
202 20 s per point (refer to the SI for additional information regarding this
203 measurement).

204 Time-resolved simultaneous CRRM and CA measurements were conducted
205 by continuously collecting RR spectra from a single biofilm location $5\text{ }\mu\text{m}$ in
206 depth from the electrode surface. During the tests, the potential of the working
207 electrode was stepped from an initial value (E_i) to a final value (E_f) to perturb
208 the original redox equilibrium E_i . While E_i was set at -0.5 V , E_f of -0.2 V , 0 V ,
209 and $+0.2\text{ V}$ where used. The E_i was kept for a total of 300 s, after which the
210 potential was instantaneously shifted to the E_f and kept for additional 240 s.
211 The subsequent relaxation profiles of current and intensity of the Raman
212 vibrational mode ν_{15} at about 750 cm^{-1} (used as marker for the redox state of

213 OMCs²⁰) was used to monitor the redox processes occurring during the
214 potential step experiment. To avoid over exposure of the biofilm to the laser
215 beam, the collection of the RR spectra started at $t = 240$ s, that is, 60 s before
216 the transition, and continued for the remaining of the test. Spectral acquisition
217 was done at integration time of 0.2 s. To further confirm the redox state of the
218 probed OMCs, additional experiments were carried out where single spectra
219 were collected during 60 s prior to the transition, and then again 180 s after
220 the transition. The integration time for these tests was 60 s.

221 **Results and Discussion**

222 **Analysis of the voltammetric response of electroactive biofilms**

223 Biofilms were incubated at a potential of 0 V until stable catalytic substrate
224 oxidation was achieved as indicated by current profiles (data not shown).
225 Confocal laser scanning microscopy (CLSM) micrographs using Fluorescent
226 *in situ* Hybridization (FISH) labeling showed the electroactive organisms
227 forming a homogeneous coverage of the electrode surface with an average
228 thickness of (17 ± 4) μm (Figure S5). Analysis of the individual cell clusters
229 scraped off the electrode confirmed the presence of *Geobacter spp.* (Text
230 S2). The voltammetric response of the biofilms was monitored under both
231 turnover and non-turnover conditions, that is, in the presence and in the
232 absence of the metabolic substrate acetate, respectively. Typical
233 voltammograms are reported in Figure 1. Measurements in turnover
234 conditions revealed the presence of one catalytic redox-active site E^f centered
235 at -0.350 V. This value is very close to the arithmetic average ($E_{1/2}$ at -0.346
236 V) of the two redox couples $E^{f,1}$ and $E^{f,2}$ obtained under non-turnover
237 conditions and centered at formal potentials of -0.389 V and -0.303 V,
238 respectively, thus suggesting the involvement of both redox couples in the
239 catalytic current. This voltammetric response has been typically observed in
240 *Geobacter* enriched electroactive biofilms on graphite, roughened silver and
241 glassy carbon,^{14,20,31} demonstrating that ITO represents a suitable electrode
242 material for electroactive biofilms, as previously shown by others.^{12,13,16,32,33}

243

244 **Analysis of the RR spectra of biofilms in completely reduced or oxidized**
245 **electrochemical conditions in the absence of metabolic substrate**

246 The spectroelectrochemical cells used in this study allowed for CRRM
247 measurements to be performed directly on the biofilms in their culturing media
248 without the need to expose them to air while transferring onto the microscope
249 stage. This represented a major advancement from our previous set ups.^{20,28}
250 The use of transparent ITO electrodes deposited on a glass substrate (0.5
251 mm thick cover slip), allowed for the Raman observations to be performed
252 non-invasively from the outside of the spectroelectrochemical cell, providing
253 minimal disturbance to the biofilms.

254 RR spectra of biofilms collected under potentiostatic conditions in the absence
255 of acetate are reported in Figure 2. Bands assignment is depicted in Table 1.
256 Consistently to what we reported previously, the vibrational bands ascribed to
257 c-type heme groups of the cytochromes embedded in the biofilms dominate
258 the RR spectra.²⁰ The mode ν_{15} , observed at around 750 cm^{-1} is ascribed to
259 the pyrrole breathing. The intensity of this band is proportional not only to the
260 presence of c-type hemes,³⁴ but also to the amount of cytochromes in the
261 reduced state.^{20,35-37} Modes in the mid-frequency region ($1100\text{-}1700\text{ cm}^{-1}$),
262 ascribed primarily to stretching vibrations of the porphyrin ring, are indicative
263 of oxidation-, spin-, and coordination-state of the Fe atom.³⁸ With the anode
264 poised at 0 V – hence more positive than the average of the macroscopic
265 redox potentials $E_{1/2}$ as determined by non-turnover voltammetry discussed
266 above - the modes ν_{21} , ν_4 , ν_{20} , ν_2 , and ν_{10} were centered respectively at 1316,
267 1369, 1400, 1583, and 1635 cm^{-1} and a shoulder appeared in
268 correspondence of the mode ν_{11} at 1563 cm^{-1} , consistently with an oxidized
269 heme group. Conversely, the application the negative potential of -0.6 V (*i.e.*,
270 more negative than the $E_{1/2}$) caused the modes ν_{21} , ν_4 , and ν_{20} , to downshift
271 to 1310, 1360, 1391, respectively, while the ν_3 (not resolvable at 0 V)
272 appeared at 1496 cm^{-1} . The mode ν_{10} did not shift although it reduced
273 considerably its intensity, and the mode ν_{11} was no longer resolvable. The
274 band ν_{15} also did not shift in position, but its intensity was considerably
275 enhanced by the application of the negative potential. These changes are
276 consistent with an oxidized (at 0 V) and reduced (at -0.6 V) heme group

277 having a six-coordinated iron atom in low-spin state. Even if mixed culture
278 biofilms on graphite, glassy carbon and silver display the typical His-Fe-His
279 axial ligation in both oxidation states,^{14,20,28} our data on ITO show a different
280 axial ligation at different oxidation states. In fact, while the ν_3 of the reduced
281 state is consistent with the His-Fe-His axial ligation, the ν_4 and the ν_{10} of the
282 oxidized heme at 1369 and 1635 cm^{-1} indicate the His-Fe-Met axial ligation.³⁹
283 This is the first time such a ligation is observed for mixed culture biofilms.
284 However, since CRRM spectra ascribable to the His-Fe-Met ligation were
285 reported by others for oxidized *G. sulfurreducens* biofilms on ITO,¹⁶ we argue
286 that the electrode material we used contributed to select those bacterial
287 species displaying the same axial ligation as *G. sulfurreducens* on the same
288 electrode material. This observation reinforces the conclusions of a recent
289 report on the impact of surface composition on the redox properties of
290 microbial biofilms.⁴⁰

291

292 **Measurements of the redox state of cytochromes across biofilm sections in the** 293 **presence of metabolic substrate**

294 The RR spectra recorded under non-turnover condition at the extreme
295 potentials of -0.6 V and 0 V will serve as reference spectra for the reduced
296 and the oxidized OMCs, respectively. In fact, the spectra discussed in this
297 study do not deviate from those shown in Figure 2 significantly. For this
298 reason, a qualitative assignment of the dominant redox state is possible by
299 tracking the spectral position of redox-sensitive vibrational modes ν_{15} , ν_{21} , ν_4 ,
300 ν_{20} , ν_3 , and ν_{10} , without the need to adopt laborious fitting procedures. The
301 knowledge of the redox state of OMCs in actively respiring biofilms have been
302 subject of intense research in recent years, since it provides important
303 information on the rate-limiting steps controlling the catalytic current
304 generation in electroactive biofilms. According to the redox conduction model
305 developed by Strycharz and coworkers for a system not limited by ET at the
306 biofilm/electrode interface (that is, a system where the ET rate constant for
307 heterogeneous electron transfer, k_{het} , is very large), in the presence of
308 oxidizing electrode potential, the RR spectra of a metabolizing biofilm is
309 expected to include the contribution of oxidized cytochromes, and the

310 establishment of a measurable redox gradient.¹⁸ This was recently reported
311 for *G. sulfurreducens* biofilms studied with confocal Raman spectroscopy.^{11,16}

312 To study possible local changes of redox state for OMCs in our mixed
313 community biofilms, we used the confocal capabilities of our system to collect
314 RR spectra from different focal planes within the biofilms. We performed
315 measurements with the anode poised at the potentials of 0 V and 0.2 V, and
316 in the presence of different levels of acetate (*i.e.*, 1 mM, 5 mM, and 20 mM).
317 Each spectrum was acquired sequentially every 5 μm across a 60 μm long
318 line extending in the Z-direction (starting from the electrode surface), using an
319 integration time of 20 seconds. An example of a redox profiling measurements
320 in the presence of 20 mM acetate and with the anode poised at 0.2 V is
321 included in the SI, together with additional details on the measurement (Text
322 S1). Each spectrum was evaluated individually to assess the position of the
323 redox marker bands ν_{21} , ν_4 , and ν_{20} , which are ascribed to the largest spectral
324 shifts upon changes in redox state (*vide supra*). Per each set of experiments,
325 the respective spectral positions were grouped and averaged per discrete
326 depth step (5 μm). Actual values are summarized in Table S1 and S2 in the
327 SI, while Figure 3 shows a visual representation of the results. In fact, the
328 figure depicts the average positions of the modes ν_{21} , ν_4 , and ν_{20} at increasing
329 distances from the electrode surface. In order to assist the assessment of the
330 redox state, the figure also reports the average position of the bands ν_{21} , ν_4 ,
331 and ν_{20} recorded in non-turnover conditions with the anode poised at 0 V
332 or -0.6 V (attributed to OMCs in completely oxidized or reduced state,
333 respectively), thus depicting the full range of variations for these redox
334 markers upon shifts in redox state. Results in Figure 3 show that in the
335 presence of non-limiting levels of metabolic substrate, that is at 5 and 20 mM
336 sodium acetate, and with the electrode poised at 0 V, cytochromes are
337 observed mostly in the reduced redox state. This is supported by the position
338 of the redox markers ν_{21} , ν_4 , and ν_{20} centered at around 1311 cm^{-1} , 1360 cm^{-1} ,
339 and 1392 cm^{-1} (Table S1), which match closely the position of the same
340 marker bands as recorded in the absence of metabolic substrate and with the
341 electrode poised at -0.6 V, conditions at which cytochromes are almost
342 completely reduced (see Table 1). Interestingly, the position of the marker

343 bands is virtually identical at any distance from the electrode surface,
344 suggesting the absence of a measurable redox gradient across the biofilms,
345 even in proximity to the electrode. Increasing the driving force by poisoning the
346 electrode potential to 0.2 V did not result in appreciable changes in the
347 redox state or in the appearance of a measurable redox gradient (Figure 3b).
348 In fact, on average the bands ν_{21} , ν_4 , and ν_{20} were centered at around of
349 1311, 1359, 1392 cm^{-1} in 5 mM acetate, and 1311, 1360, and 1392 cm^{-1} in 20
350 mM acetate (Table S1). It was not until we performed measurements in the
351 presence 1 mM of acetate in the bulk liquid that we noticed appreciable shifts
352 in position of the marker bands suggesting significant changes in the redox
353 state of cytochrome (Figure 3). In fact, in this case the relative position of the
354 bands ν_{21} , ν_4 , and ν_{20} is consistent with cytochromes in completely oxidized
355 state. Diffusional limitations for substrates within the biofilm were likely
356 irrelevant for acetate levels in the bulk liquid of 5 mM and 20 mM, as it was
357 also suggested previously.⁴¹ It is worth noting that no significant increase of
358 steady-state current followed the increase in the acetate concentration from 5
359 to 20 mM at both employed potentials (Figure S6), confirming that the whole
360 biofilm performed at the maximal allowed conversion rates and therefore,
361 local variations of the respiration rates due to substrate limitations can be
362 excluded.

363

364 **Analysis of electron transfer kinetics**

365 The results presented above, specifically, the fact that the oxidation of OMCs
366 lags significantly behind changes in electrode potential, and the absence of a
367 measurable concentration gradient of oxidized (and reduced) cytochromes
368 across the biofilms under turnover conditions, suggests that the limiting step
369 for the electron transfer process in the electroactive microbial community
370 studied here may lay at the biofilm/electrode interface, contrary with what has
371 been observed also using CRRM on *G. sulfurreducens* films grown on gold,¹¹
372 and ITO electrodes.¹⁶ In these studies the presence of a measurable redox
373 gradient suggested that the step that limits the overall electron transport is the
374 long-range, homogeneous ET, as opposed to a very fast heterogeneous ET at
375 the biofilm/electrode interface. Currently accepted models for electron transfer
376 across a conductive biofilm matrix describe the catalytic acetate oxidation as

377 the combination of numerous processes, each accounting for a particular
378 electron transfer step.^{18,42} Steps include 1) the mass transport of acetate into
379 the microbial cells, 2) its internal turnover and 3) the reduction of ET
380 mediators (identified here as OMCs) (steps 1 to 3 in Strycharz *et al.*¹⁸);
381 followed by 4) homogeneous electron transfer through the biofilm matrix via a
382 sequence of ET reactions between fixed mediators (*e.g.*, electron hopping),
383 and 5) the final heterogeneous electron transfer to the electrode by the
384 oxidation of mediators at the biofilm/electrode interface (steps 4 and 5 in
385 Strycharz *et al.*¹⁸). When used to describe catalytic acetate oxidation of *G.*
386 *sulfurreducens* biofilms, this model identified non-limiting heterogeneous
387 electron transfer kinetics (step 5 above), while identifying the long-range
388 electron transfer across the biofilm matrix (step 4) and metabolic substrate
389 conversions (steps 1 to 3) as the main rate-limiting steps.^{18,42} Conversely,
390 results presented recently by others, suggest that differences might exist
391 between mixed and pure culture electroactive biofilms in the identity of the
392 rate-limiting ET steps. For example, using time-resolved surface-enhanced
393 resonance Raman scattering (SERRS), Ly *et al.*⁴³ probed selectively the
394 redox states of the OMCs at the biofilm/electrode interface, reporting that the
395 heterogeneous ET was a very slow process (having a $k_{ET} = 0.03 \text{ s}^{-1}$) that was
396 coupled with a slightly faster long-range ET (homogeneous) having a
397 predicted rate constant k_{hom} of 1.2 s^{-1} .

398 To provide a deeper insight into the ET rates of mixed culture biofilms,
399 we applied a combined experimental approach consisting of monitoring the
400 changes of oxidation states of the OMCs in time by means of CRRM during
401 the application of a potential step from an initial (E_i) to a final potential (E_f), the
402 latter process being probed by chronoamperometry (CA). We used the
403 subsequent relaxation of the current profile as indicative of all redox
404 processes occurring in the bulk biofilm and at the biofilm/electrode interface,
405 while we used the relaxation of the intensity of the RR spectra binned at the
406 band ν_{15} at 750 cm^{-1} to monitor long-range electron transfer involving
407 homogeneous transfer between OMCs at a portion of the biofilm at a specific
408 distance from the electrode (fixed at $5 \mu\text{m}$ from the electrode surface on all
409 measurements). The choice of the marker band ν_{15} at 750 cm^{-1} was dictated

410 by its relation to the amount of cytochromes in the reduced state. We used a
411 similar approach previously to monitor dynamic variations of heme redox-state
412 during combined voltammetry and RR measurements.²⁰

413 Figure 4 reports typical measurements performed using a potential step with
414 amplitude of 0.7 V from an initial E_i of -0.5 V (which was maintained for 300 s
415 prior to the transition) to a final E_f of +0.2 V (maintained for 240 s after the
416 transition). The E_i was intentionally chosen lower than the average $E_{1/2}$ as
417 determined by non-turnover voltammetry and equal to -0.346 V (*vide supra*),
418 to initiate the transition from a largely reduced redox state of the OMCs. The
419 CA traces of current vs time in the absence of acetate after the transition ($t >$
420 300 s) display an exponential relaxation phase which levels off towards zero
421 (Figure 4b). Conversely, in the presence of acetate the current traces display
422 a fast discharge process in the instants immediately after the transition (within
423 a few seconds past $t = 300$ s), which is then superimposed by an electron
424 producing process that levels off towards an average steady-state catalytic
425 current of (41.8 ± 0.1) μA in the later stages of the measurements (Figure 4a).
426 Interestingly, analogous profiles were obtained also during CA tests using a
427 final E_f of 0 V, hence a smaller driving force (Figure S7). Busalmen and
428 coworkers obtained similar profiles of current vs time on *G. sulfurreducens*
429 grown on graphite electrodes under turnover conditions, albeit with different
430 time spans.^{42,44} The authors described the turnover current as due to the
431 simultaneous contribution of two processes: 1) the reoxidation of the
432 cytochromes reduced in the phases preceding the transition, which is
433 responsible for the initial fast discharge, and 2) the current deriving from the
434 metabolism of acetate, which lags behind the first process, at least for the first
435 instants after the transition, probably due to the requirement for acetate to be
436 transported into the microbial cells before being metabolized. Using a similar
437 conceptual approach, we can consider the turnover current (i_{turnover}) as due to
438 the combination of all electron transfer steps (*i.e.*, steps 1 to 5 in Strycharz *et*
439 *al.*¹⁸), while the non-turnover current ($i_{\text{non-turnover}}$) as due to the combination of
440 homogeneous and heterogeneous ET alone because of the absence of
441 acetate in the medium (*i.e.*, steps 4 and 5 in Strycharz *et al.*¹⁸). Therefore, the
442 shape of the difference curve $i_{\text{turnover}} - i_{\text{non-turnover}}$ accounts for the contributions

443 of acetate metabolism alone (Figure 5). The shape of this amperometric trace
444 accounts for steps 1 to 3 in Strycharz *et al.*,¹⁸ that is, acetate uptake into the
445 microbial cells, its metabolic oxidation, and ET to the matrix OMCs. The
446 analysis of this amperometric profile suggests that the discharge current
447 prevails over the metabolic current for a very short time after the transition. At
448 about $t = 301$ s – thus much earlier than the complete oxidation of the OMCs
449 occurs – the current increases due to the microbial metabolism of acetate.
450 The trace also displays the presence of a local maximum at about 309 s (that
451 is 9 s after the transition). It is possible that this maximum is due to the
452 electrons generated by the oxidation of the acetate that had already entered
453 the microbial cells in the phases prior the transition, when the potential was
454 more negative than that required for the catalytic acetate oxidation. After this
455 internal pool is exhausted (at $t > 309$ s), however, the production rate of
456 additional electrons will depend on the rate-limiting steps between the
457 transport of new acetate into the cells, its subsequent oxidation, and ET to
458 OMCs. This process is probably regulated by the internal metabolism of the
459 microbes, since changes in gene expression are unlikely within the
460 timeframes observed. Hence, is possible that the presence of the minimum in
461 electric current output at about 319 s is due to the initial need by the
462 organisms to sense the change in potential, and adjust the internal metabolic
463 machinery to the new redox conditions as imposed by the transition to E_f .
464 Recent findings reporting the ability of metal-respiring bacteria to sense the
465 electric field – a property called electrokinesis – forces us to consider that this
466 possibility occurs also in the case of our mixed cultures.⁴⁵
467 Examination of the CRRM spectra corroborates the interpretation provided
468 above. First of all, analysis of the intensity of the band ν_{15} ($I_{\nu_{15}}$) during the
469 transition experiments (also reported in Figure 4) shows that prior to the
470 potential step ($t < 300$ s) under both turnover and non-turnover conditions, the
471 redox state of OMCs is mostly reduced. This is inferred by the high intensity of
472 the band ν_{15} (Figure 4a and 4b), as well as by the position of the redox
473 markers ν_{21} , ν_4 and ν_{20} in the RR spectra collected during the 60 seconds
474 prior the application of the transition (spectrum 1 and 3 in Figure 4c and 4d),
475 which proves that the transition starts with OMCs mostly in the reduced state.

476 This is not surprising since OMCs can store negative charge from the
477 intracellular metabolism under conditions at which the anode cannot act as
478 the electron sink.^{42,44,46,47} Our data show that the cell metabolism refills the
479 OMCs before their complete discharge occurs at the electrode. In other
480 words, steps 1-3 are faster than steps 4-5. Our CRRM data confirm this
481 interpretation. In fact, upon the application of the oxidizing potential E_f (+0.2
482 V), the profile of $I_{\nu_{15}}$ vs time under non-turnover condition relaxes towards
483 zero very quickly, indicating a shift in the redox state of the cytochromes at 5
484 μm from the electrode surface to mostly the oxidized state. This is confirmed
485 by the shifts of the bands ν_{21} , ν_4 and ν_{20} to 1314, 1367, 1401 cm^{-1} ,
486 respectively, as well as by the presence of the band ν_{10} at 1634 cm^{-1} (Figure
487 4d). This observation is coherent with the complete discharge of the OMCs in
488 the absence of acetate (*vide supra*). Conversely, under turnover conditions,
489 instead of the exponential decay as displayed in the absence of acetate, the
490 profiles of the $I_{\nu_{15}}$ vs time show a very sharp initial drop occurring within
491 seconds after the transition (Figure 4a), after which, instead of relaxing to zero
492 as observed in non-turnover, the $I_{\nu_{15}}$ rests at an intermediate value $\neq 0$ for the
493 remaining of the observations, consistent with an incomplete discharge of the
494 OMCs. Analysis of individual RR spectra collected after the transition, in
495 particular, position and intensity of the bands ν_{21} , ν_4 , ν_{20} , and ν_3 (virtually
496 unchanged after the transition), and the absence of the band ν_{10} confirmed
497 the redox state of the OMCs as predominantly reduced, in spite of the partial
498 reduction of intensity of the ν_{15} band (spectra 1 and 2 in Figure 4c). The initial
499 drop observed in the $I_{\nu_{15}}$ is likely ascribed to the initial discharge that prevails
500 in the instants right after the transition over the electrons produced by the
501 microbial metabolism, as discussed earlier. However, after this initial phase,
502 electrons from the microbial metabolism will feed to the bulk OMCs, keeping
503 them (mostly) in the reduced redox state. The absence of a transient phase in
504 the $I_{\nu_{15}}$ profile similar to that observed for the profile of i_{turnover} (and the i_{turnover}
505 $- i_{\text{non-turnover}}$) before reaching steady-state, suggests that the two processes
506 are not perfectly coupled. Even if the reason for this is unknown, we cannot
507 exclude the contributions of other redox mediators promoting the electron
508 transfer and thus increasing the electrocatalytic current.

509 Overall, these observations are in line with a scenario whereby in the
510 presence of acetate (and under potentiostatic conditions), electrons are
511 transferred from the microbial central metabolisms to the external OMCs at a
512 rate that is faster than the rate at which electrons travel across the conducting
513 biofilm matrix through hopping between adjacent OMCs (that is, the
514 homogeneous ET as defined above). This scenario requires the rate constant
515 for homogeneous ET k_{hom} to be smaller than the rate constant for the
516 combined metabolic turnover rates k_{mic} (which would be the rate-limiting step
517 between acetate diffusion, acetate oxidation, and electron transfer to the
518 OMCs), that is $k_{\text{mic}} > k_{\text{hom}}$. In fact, a scenario characterized by sluggish
519 microbial kinetics combined to a fast homogeneous transfer (*i.e.*, $k_{\text{mic}} < k_{\text{hom}}$)
520 is not consistent to the presence of cytochromes mostly in the reduced redox
521 state as we observed during the transient CA test under turnover conditions
522 ($I_{V_{15}}$ trace in Figure 4a). Under these conditions, the rate-limiting process for
523 ET would be either the homogeneous ET or the heterogeneous ET, or the
524 combination of both. Previous analysis by Ly *et al.* suggested that the bulk ET
525 is faster than the interfacial process ($k_{\text{hom}} > k_{\text{het}}$). This is consistent with our
526 measurements (which cannot exclude, however, $k_{\text{hom}} \approx k_{\text{het}}$). In fact, a
527 scenario consisting of a sluggish ET in the biofilm and a much faster
528 interfacial electron transfer (*i.e.*, $k_{\text{hom}} \ll k_{\text{het}}$) would not have made possible
529 for the bulk OMCs as probed by CRRM to be completely oxidized during the
530 CA test in non-turnover conditions, and it would have resulted in the
531 generation of an appreciable concentration gradient of oxidized OMCs within
532 the biofilms, consistent to CRRM observations on *G. sulfurreducens*.^{11,16} Both
533 our measurements at steady-state and during the transient CA experiments
534 do not seem to support such a scenario (*vide supra*). CRRM measurements
535 performed on biofilms comprised of individual cell clusters, which therefore
536 exclude the contribution of multiple cell layers to the redox state of the OMCs
537 in proximity of the interface, are also consistent to the proposed scenario of a
538 sluggish heterogeneous ET (data not shown).

539

540 **Analysis of the apparent electron transfer rate constant for homogeneous ET**

541 Contrary to the method by Ly *et al.* based on SERRS, our approach based on
542 CRRM does not allow for the direct measurement of the interfacial ET process

543 due to the physical impossibility to focus the laser beam only on the interfacial
544 OMCs: in fact, being the thickness of the beam typically in the micrometer
545 range, it exceeds by far the thickness of the surface-confined OMCs (< 10
546 nm). Discerning the contribution of the homogeneous ET from the
547 heterogeneous ET from the current vs time trace in non-turnover conditions
548 was also not possible, since the current traces results from the contribution of
549 all ET processes in the whole biofilm.⁵ However, we focused here on the
550 examination of the long-range ET process using the analysis of the relaxation
551 profiles of the I_{V15} vs time after the transition. In fact, the relaxation constant
552 for the biofilm-embedded OMCs during the transient CA tests described
553 above can be determined by fitting of a single exponential decay function to
554 the profile of the I_{V15} vs time. By applying different E_f , it was possible to
555 determine the apparent k for homogeneous ET relatively to different driving
556 forces (ΔV). The fitting exercise yielded k_{hom} of (0.038 ± 0.004) , (0.043 ± 0.021) ,
557 and (0.066 ± 0.026) s^{-1} (averages \pm standard deviation for triplicate
558 measurements) for the potential steps with E_f of -0.2 V, 0 V, and +0.2 V.
559 Results are presented in Figure 6. Best fitting of the data was achieved with
560 an exponential function (coefficient of determination $R^2 = 0.93$). The
561 theoretical k value determined by imposing the condition of zero overpotential
562 to the fitting curve (that is, for a hypothetical potential step where $E_f = E_{1/2}$
563 = -0.346 V (see Figure 1)) is, according to the Butler-Volmer formalism, the
564 rate constant at equilibrium, k_{hom}^0 (Figure 6). The analysis yielded a k_{hom}^0 of
565 0.028 ± 0.069 s^{-1} (mean \pm 95% confidence interval). This is 60 times lower than
566 that predicted by Ly and coworkers under the hypothesis that long-range
567 electron transfer is promoted by only OMCs in the bulk biofilm,⁴³ and may
568 indicate the contribution of redox mediators other than OMCs to the
569 long-range ET. In our systems, comparison of the measured with the k_{hom} with
570 the hypothetical profile of $k_{\text{hom}}^{\text{BV}}$ vs ΔV as predicted by the Butler-Volmer
571 equation for the same value of k_{hom}^0 shows that the observed homogeneous
572 ET is indeed much slower than that predicted by the model (see Figure S8 in
573 the SI). While a weak dependency of ET rates with driving force is
574 characteristic of electron transfer via a hopping mechanism⁴⁸ and it was used
575 previously to establish the dominance of inelastic hopping over

576 tunneling,^{43,49,50} our results can be considered consistent to the simultaneous
577 interplay of a fast ET process with a much slower ET mechanism. While the
578 slow ET process could still be ascribed to hopping between adjacent
579 cytochromes, alternative fast ET pathways can perhaps include tunneling
580 between cytochromes with more favorable orientation and/or at closer
581 proximity with each other, or the involvement of alternative ET modes such as
582 bacterial nanowires with metallic-like conducting properties as proposed for
583 networks of *G. sulfurreducens*.⁵¹ It is important to note that the large signal-to-
584 noise ratio that characterizes the real-time CRRM measurements (see for
585 example the I_{V15} profiles in Figure 4, 6 and S7) may not permit to discriminate
586 with sufficient accuracy between fast and slow ET processes involving OMCs
587 (*i.e.*, the I_{V15} traces account for the macroscopic redox response of the probed
588 sample). On the other hand, the lack of additional information on the biofilm
589 architecture and composition does not permit to be resolute on this aspect at
590 this stage. For example, CRRM is not capable to detect the presence of
591 cellular components such as nanowires or other potentially important redox
592 active molecules in the biofilms examined unless they generate a detectable
593 Raman signal.

594 **Conclusions**

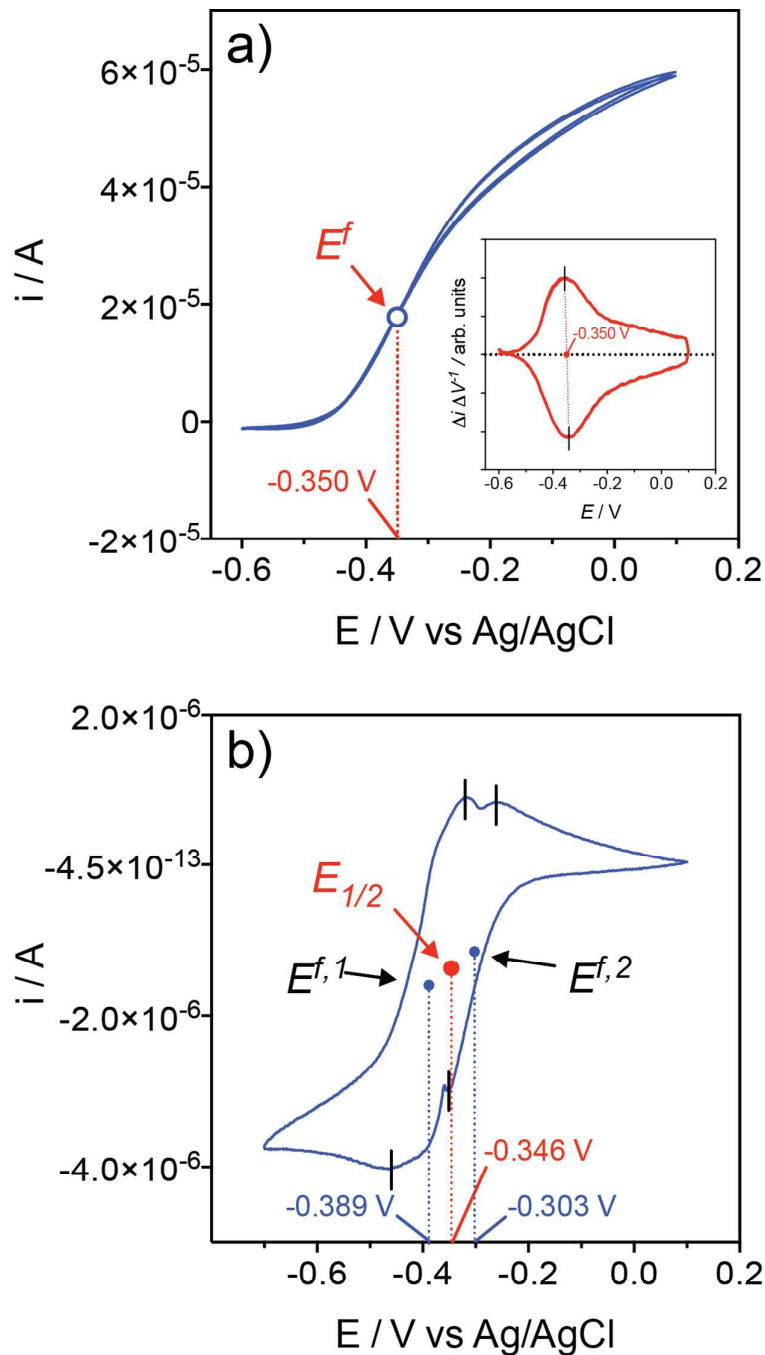
595 By using a specifically designed spectroelectrochemical cell that utilized
596 transparent ITO electrodes, it was possible to assess the redox chemistry of
597 mixed culture electroactive biofilms *in situ* and *in vivo*. Our results show that in
598 the presence of non-limiting levels of metabolic substrate, the oxidation state
599 of OMCs embedded in the biofilm matrix lags significantly behind the
600 electrode potential (contrary to measurements in substrate-depleted medium).
601 Under the same conditions, the redox state of OMCs is mostly reduced at any
602 distance from the electrode surface, even when the electrode is poised at
603 potentials sufficiently high to determine complete oxidation of the OMCs in the
604 absence of substrate. CRRM analysis during potential step transitions under
605 turnover and non-turnover conditions suggested that the respiration rate is a
606 fast process compared to both long-range and interfacial electron transfer
607 processes. Thus, in mixed community biofilms grown on ITO, a fast metabolic

608 acetate respiration feeds electrons to the OMC coupled with much slower or
609 comparable homogeneous ET between OMCs in the conducting biofilm
610 network, and heterogeneous electron transfer at the interface.

611 **Acknowledgements**

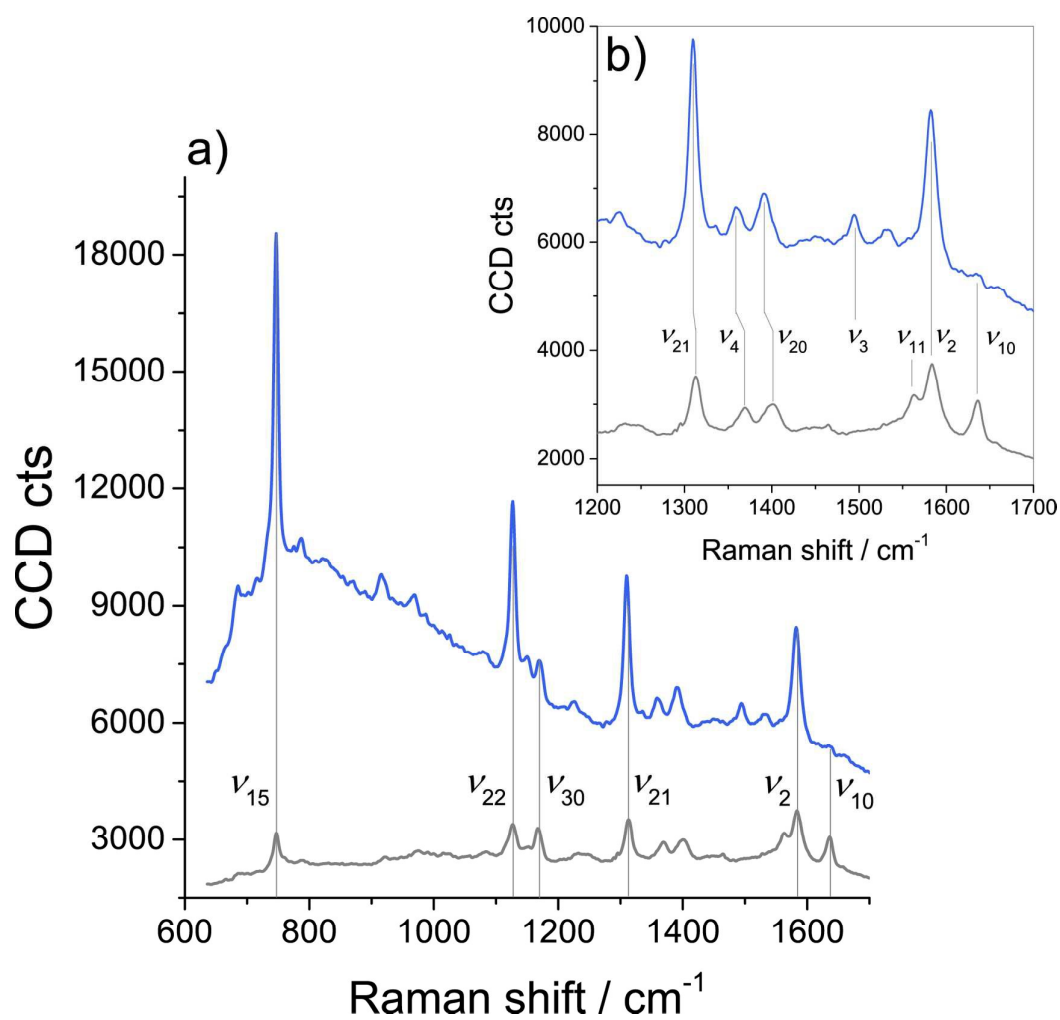
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620 Figures and Tables



621

622 **Figure 1.** Typical cyclic voltammograms on the electroactive biofilms recorded at the scan
 623 rate of 1 mV s^{-1} . a) Turnover CV recorded in the presence of acetate (20 mM), where E^f
 624 indicates the putative electron transfer site centered at -0.364 V . Insert indicated the first
 625 derivative of the turnover CV. b) Non-turnover CVs in acetate-depleted medium, where $E_{1/2}$
 626 centered at -0.346 V indicates the arithmetic average of two redox couples $E^{f,1}$ and $E^{f,2}$.



627

628 **Figure 2.** RR spectra of electroactive biofilms in acetate-depleted medium (average of629 multiple measurements) in a) the spectral region between 600 and 1700 cm^{-1} and b)630 magnification on the region between 1200 and 1700 cm^{-1} . RR spectra were recorded with the

631 anode potential poised at -0.6 V (blue line) and 0 V (grey line). Relative positions of the

632 marker bands indicated in the figure are reported in Table 1.

633

634

635 **Table 1.** Normal mode assignment of the most prominent bands from the averaged Raman
 636 spectra obtained with the working electron poised at 0 V and -0.6 V shown in Figure 2.

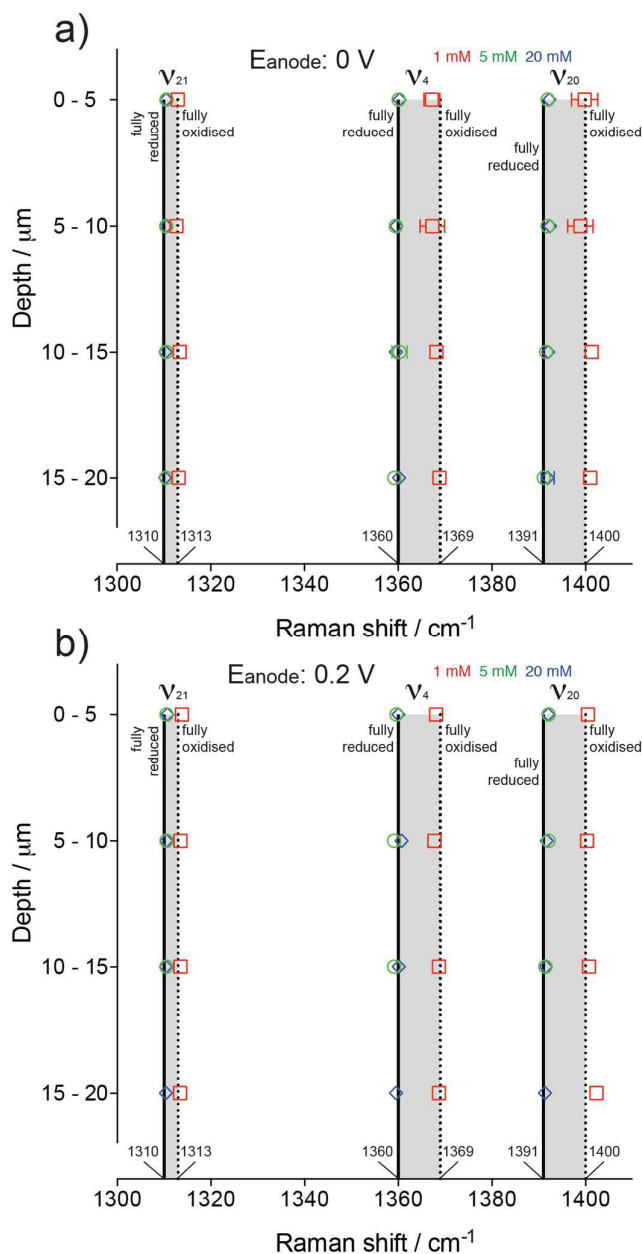
Band assignment ^a	RR bands at 0V (cm ⁻¹)	RR bands at -0.6V (cm ⁻¹)
ν_{15}	747	746
ν_{22}	1127	1127
ν_{30}	1167	1165
ν_{21}	1313	1310
ν_4	1369	1360
ν_{20}	1400	1391
ν_3	-	1494
ν_{11}	1563 ^b	-
ν_2	1583	1582
ν_{10}	1635	1635

^a assignment accordingly to Hu *et al.*⁵²

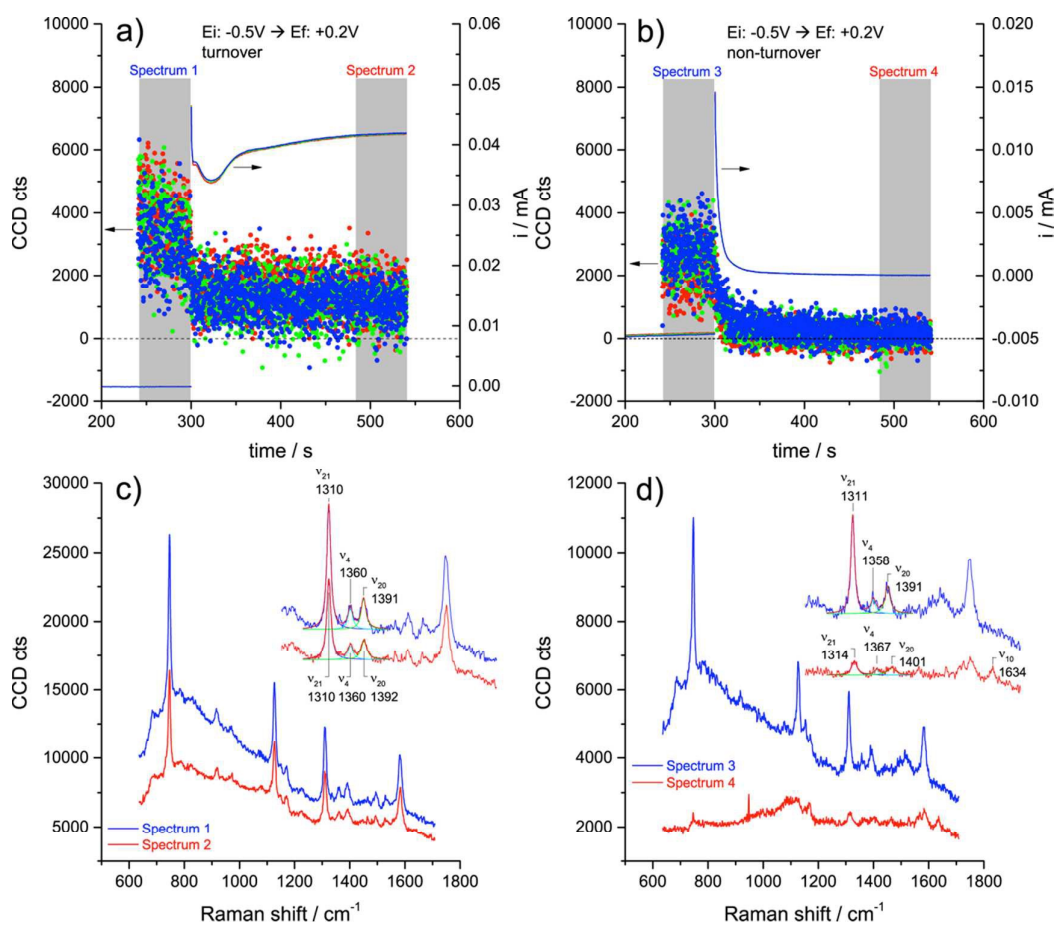
637 ^b shoulder.

638

639



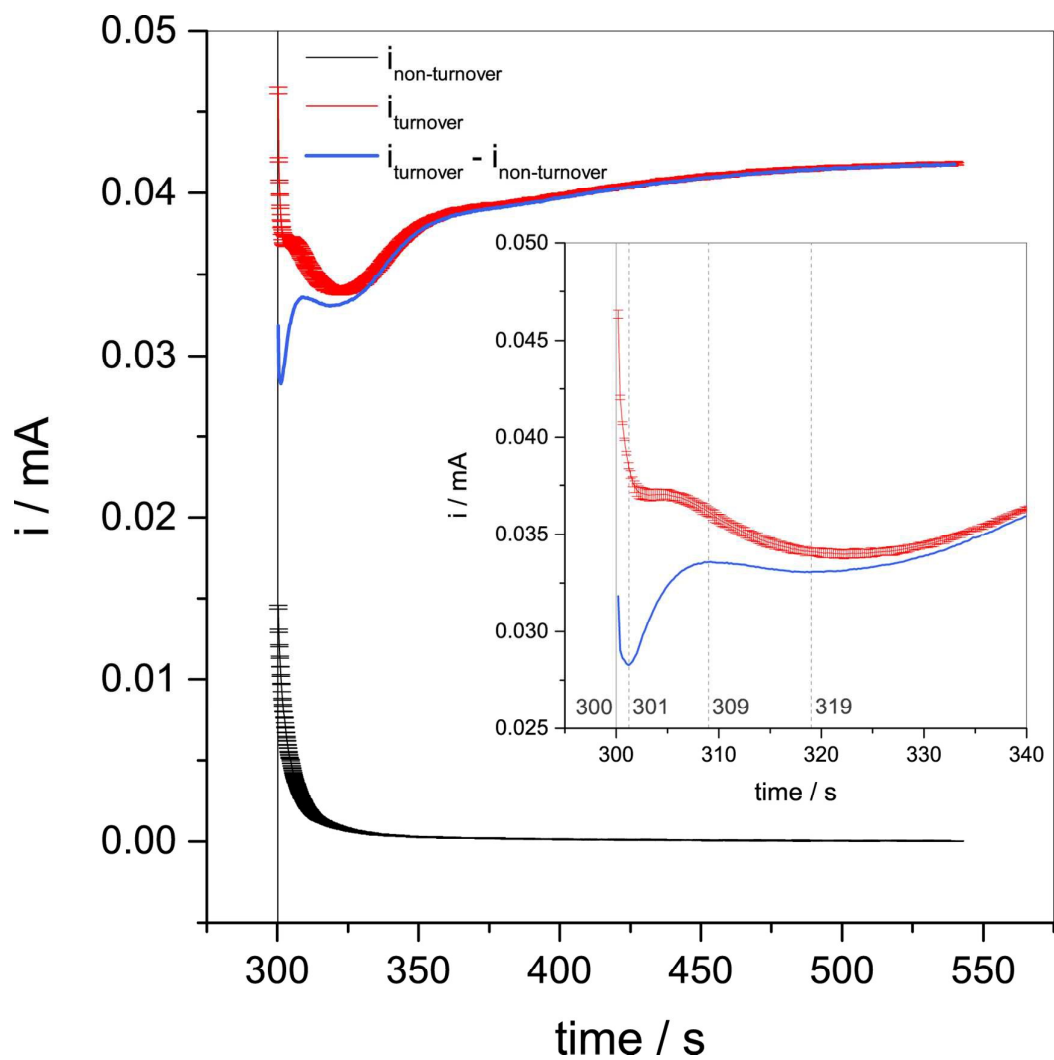
640
 641 **Figure 3.** Depth profiling under turnover conditions in media containing acetate: 1 mM (red
 642 squares), 5 mM (green circles), and 20 mM (blue diamonds). Measurements performed with
 643 the working electrode poised at 0V or +0.2V. The symbols depict the position of the redox
 644 markers ν_{21} , ν_4 , and ν_{20} as a function of the depth position. RR spectra were collected as 12
 645 single spectrum acquisitions along a 60 μm line in the Z direction with an integration time of
 646 20 s per point. Points were then grouped in discrete intervals of 5 μm and averaged (refer to
 647 the SI for details). Note that point Z = 0 μm corresponds to the biofilm/ITO interface,
 648 determined as described in the SI. Note that symbols for the data at 5 and 20 mM overlap in
 649 some instances. Standard deviations values are in many instances smaller than the symbols,
 650 hence, average and standard deviations are also reported in Tables S1 and S2 in the SI.



651

652 **Figure 4.** CRRM and Chronoamperometry measurements for potential step with $E_i = -0.5\text{ V}$ to
 653 $E_f = +0.2\text{ V}$. a) Current vs time and intensity of the band ν_{15} vs time during the test under
 654 turnover and b) non-turnover conditions. c) and d) RR spectra recorded during a 60 s
 655 accumulation before the transition (spectra 1 and 3) and 3 minutes after the transition
 656 (spectra 2 and 4) during c) turnover and d) non-turnover measurements. Inserts in c) and d)
 657 show magnification to the redox markers region and shows relative position of the bands ν_{21} ,
 658 ν_4 , ν_{20} , and ν_{10} .

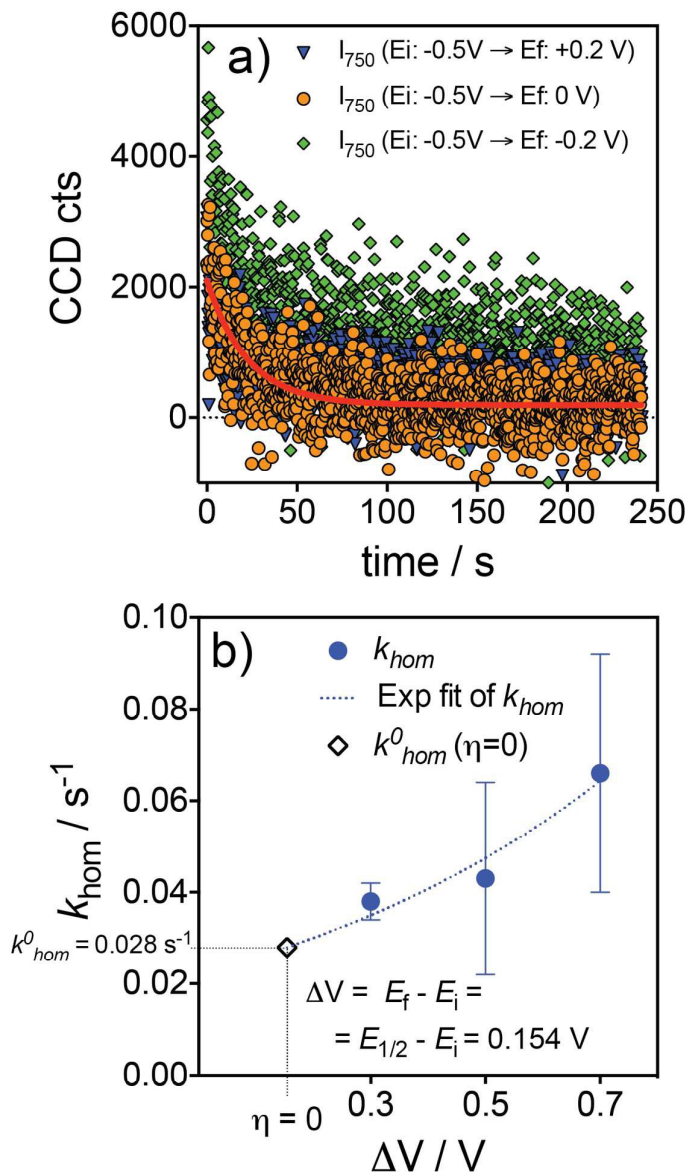
659



660

661 **Figure 5.** Turnover (i_{turnover}) and non-turnover ($i_{\text{non-turnover}}$) current vs time traces (averages and
662 standard deviations of the three CA profiles reported in Figure 4) for a potential step with $E_i =$
663 -0.5 V to $E_f = +0.2$ V. The calculated trace $i_{\text{turnover}} - i_{\text{non-turnover}}$ represents the current due to
664 acetate metabolism alone (see text).

665



666

667 **Figure 6.** Chronoamperometry performed in non-turnover conditions. a) Relaxation profiles of
 668 the intensity of the band ν_{15} vs time after the transition from E_i to E_f where $E_i = -0.5\text{V}$ and $E_f =$
 669 -0.2V , 0V , and $+0.2\text{V}$. Values of k_{hom} were evaluated by fitting the relaxation profile of the
 670 intensity of the redox marker mode ν_{15} with a monoexponential decay function (an example
 671 for $E_f = 0\text{V}$ is indicated in the figure). b) Dependence of the apparent electron-transfer rate
 672 constants for homogeneous ET (k_{hom}) on the driving force ($\Delta E = E_f - E_i$). Values are reported
 673 as means \pm standard deviations for triplicate measurements. The values of k_{hom} as a function
 674 of the driving force were fit with an exponential function to determine the k_{hom} at zero
 675 overpotential ($\eta=0$) - that is, for an hypothetical step where $E_f = E_{1/2} = -0.346\text{V}$ - which
 676 corresponds to the rate constant at equilibrium, k_{hom}^0 . The fitting yielded $k_{\text{hom}}^0 = (0.028 \pm 0.069)$
 677 s^{-1} (average \pm 95% confidence interval).

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