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## Microbial characterization of anode-respiring bacteria within biofilms developed from cultures previously enriched in dissimilatory metal-reducing bacteria

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1 Microbial characterization of anode-respiring bacteria within biofilms  
2 developed from cultures previously enriched in dissimilatory metal-  
3 reducing bacteria

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13

14 **Abstract**

15 To date, most of the enrichment methods aiming at the growth of an efficient electroactive  
16 biofilm are based on the hypothesis that anode respiring bacteria (ARB) are mostly  
17 dissimilatory metal-reducing bacteria (DMRB). Thus, this work evaluated the use of a culture  
18 enriched in DMRB as a strategy to enrich ARB on anodes. DMRB were enriched with Fe(III)  
19 as final electron acceptor and then transferred to a potentiostatically-controlled system with an  
20 anode as sole final electron acceptor. Three successive iron-enrichment cultures were carried  
21 out. The first step of enrichment revealed a successful selection of the high current-producing  
22 ARB *Geoalkalibacter subterraneus*. After few successive enrichment steps, the microbial  
23 community analysis in electroactive biofilms showed a significant divergence with an impact  
24 on the biofilm electroactivity. Enrichment of ARB in electroactive biofilms through the pre-  
25 selection of DMRB should therefore be carefully considered.

26 **Keywords:**

27 Microbial bioelectrochemical systems; Dissimilatory metal-reducing bacteria; Anode  
28 respiring bacteria; *Geoalkalibacter subterraneus*.

## 29 **1 Introduction**

30 Bioelectrochemical systems (BES) such as microbial fuel cells (MFC), microbial  
31 electrolysis cells (MEC) or, more recently, microbial electrosynthesis (MES) reactors rely on  
32 the ability of electroactive bacteria to transfer/accept electrons to/from a solid external  
33 electron acceptor/donor such as an anode or a cathode, respectively. The transferred electrical  
34 current can be used for the direct production of electricity in MFCs or the production of  
35 valuable molecules via MES.

36 In previous studies, it has been demonstrated that ARB can be enriched from many  
37 different environmental sources such as freshwater, marine sediments, salt marshes, anaerobic  
38 sludge, wastewater treatment plant sludge and mangrove swamp sediments (Miceli et al.,  
39 2012). In natural environments such as aquatic sediments, submerged soils and the terrestrial  
40 subsurface, dissimilatory metal-reducing bacteria (DMRB) use a variety of extracellular  
41 electron acceptors like iron and manganese (Lovley, 1993). Interestingly, many DMRB are as  
42 well capable of using a conductive material as final electron acceptor. Thus, such ability in  
43 DMRB has opened a window of opportunities in the specific enrichment of ARB (Wang et  
44 al., 2010).

45 Different enrichment techniques have been used to form high current-producing biofilms  
46 such as the 1) use of effluents from continuously operated BESs (Sleutels et al., 2011), 2)  
47 scraping and dispersion of an already well formed electroactive biofilm (Liu et al., 2008) and  
48 3) placement of electrodes with developed electroactive biofilms in the vicinity of a new  
49 electrode material (Kim et al., 2005). Although the previous enrichment of DMRB as  
50 inoculum for BES has been occasionally tested, the lack of information regarding the analysis

51 of the microbial communities prohibited a detailed discussion (Kim et al., 2005; Wang et al.,  
52 2010).

53 A recent study by Feng et al. (2012) has reported the improvement of current densities by  
54 pure cultures of ARB within the *Shewanellaceae* family when such cultures have been  
55 amended with iron. On the other hand, using mixed cultures as original inoculum, only a  
56 few studies have focused on enriching DMRB bacteria in order to get a stable and efficient  
57 biofilm consortium mainly composed of ARB.

58 Zhang et al. (2014) enriched ARB by adding Fe(III) in the electrolyte solution of BESs  
59 experiments. This strategy permitted to i) select iron-reducing bacteria from wastewaters  
60 while anaerobically oxidizing organic matter and ii) a current production three times higher  
61 than the one obtained without the iron addition. Additionally, Kim et al. (2005) previously tried  
62 to enrich mixed cultures in DMRB to be further used as inoculum for BES. They conducted  
63 enrichments of DMRB contained in sludge by exposing certain amount of its biomass to a  
64 ferric iron medium. However, neither the improvement in performance or the successful  
65 enrichment of DMRB were demonstrated.

66 A more detailed study that reported the previous enrichment of DMRB as inoculum for  
67 BES is the work by Wang et al. (2010). They exposed an already developed electroactive  
68 biofilm to an iron-rich medium. Once iron reduction took place, they used such DMRB-  
69 enriched biomass as inoculum for BESs. As expected, the electrochemical performances were  
70 improved for the BESs inoculated with previous iron-enriched biomass (power density: 226  
71  $\text{mW/m}^2$ , coulombic efficiency: 34%), that was higher than the original electroactive biofilm  
72 ( $209 \text{ mW/m}^2$ , 23%). Unfortunately, no details were presented regarding the microbial  
73 selection occurred therein.

74 Until now, most of the ARB enrichment strategies have consisted in the use of an existing  
75 electroactive biofilm (Kim et al., 2005; Miceli et al., 2012; Wang et al., 2010) or the use of

76 effluent from a running BES (Sleutels et al., 2011). These techniques imply to run a BES  
77 before in order to obtain an efficient electroactive biofilm. Thus, an iron-enrichment strategy  
78 requires less energy and is a less time consuming ARB pre-selection technique.

79 The objective of the present work was to evaluate whether an enrichment procedure of  
80 DMRB is necessarily linked to the presence ARB in terms of electron transfer performances.  
81 An iron enrichment method was developed to specifically select DMRB capable of further  
82 colonize the electrode of a potentiostically-controlled system. The effect of successive iron-  
83 reducing enrichment culture stages on microbial selection and electrochemical performance  
84 of both DMRB and ARB in biofilms was then characterized. The novelty of this work is the  
85 focus on the description of microbial communities during both types of enrichments. This  
86 analysis is ignored in several studies that mainly focus on improving the overall performance  
87 of the system, without the detailed understanding of the microbial community  
88 characterization.

## 89 **2 Materials and methods**

90 The enrichment of both dissimilatory metal-reducing bacteria (DMRB) cultures and  
91 anode-respiring bacteria (ARB) embedded within biofilms was performed under moderate  
92 halophilic conditions (35 g<sub>NaCl</sub>/L) i) due to the salinity measured in the sediment sampling  
93 location (see inoculum details in supplementary material) and ii) to provide enough  
94 conductivity in BES. Sediments from a salt plant already adapted to high salt concentrations  
95 were used to i) inoculate a medium containing Fe(III) as sole electron acceptor in order to  
96 favor the selection of DMRB (E1) and ii) inoculate an electrochemical system equipped with  
97 a three-electrode setup to form an electroactive biofilm (B0) on a graphite anode (Fig. S1).  
98 The first DMRB-enrichment culture named E1 was then used as inoculum for further DMRB-  
99 (E2) and ARB-enrichments (B1). This procedure was reiterated three times in four replicates  
100 to observe the effect of the succession of enrichment culture stages on both

101 bioelectrochemical performance and microbial selection. Further details on the three electrode  
102 system arrangement, DMRB and ARB enrichment cultures, chemical analyses and  
103 electrochemical data analysis are presented in the Supplementary Material.

104 Molecular analyses of bacterial communities were performed after acetate consumption  
105 in each biofilm reactor and enrichment steps. PCR products were separated by capillary  
106 electrophoresis single-strand conformation polymorphism (CE-SSCP), previously described  
107 (Pierra et al., 2015). With this technique, DNA fragments of the same size but with different  
108 DNA compositions are separated. Molecular biology protocols are detailed in the  
109 Supplementary Materials.

110

## 111 **3 Results and discussion**

### 112 **3.1 DMRB-enrichment cultures**

#### 113 **3.1.1 Iron conversion**

114 In Fig. 1A, the electron transfer from acetate to Fe(III) in DMRB-enrichments is  
115 shown as moles of iron reduced per mole of acetate. Under the tested conditions, this ratio  
116 progressively increased from 4.7 up to the stoichiometric conversion rate of 8.0  
117  $\text{mol}_{\text{Fe(II)}}/\text{mol}_{\text{acetate}}$  in DMRB-enrichments E1 and E3, respectively. Wang et al. (2010) and  
118 Roden & Lovley (1993) reported similar maximal rates in DMRB-enrichments (about 8.0  
119  $\text{mol}_{\text{Fe(II)}}/\text{mol}_{\text{acetate}}$ ). Such conversion rate is close to the maximal theoretical value of electrons  
120 transferred after complete acetate oxidation, which means that Fe(III) was the only electron  
121 acceptor available for acetate oxidation. During DMRB-enrichment steps neither biomass,  
122 liquid or gas end products were measured. Thus, it cannot be firmly excluded the existence of  
123 other metabolic pathways. The increase in acetate conversion ratio for Fe(III) reduction into  
124 Fe(II) is likely due to the progressive depletion of alternative electron acceptors that are  
125 initially present in the sediment used as inoculum.

126

-Please insert **Figure 1** here-

### 127 **3.1.2 Microbial community analysis of enriched DMRB**

128 A significant bacterial selection occurred along the DMRB-enrichment steps with the  
129 predominance of few bacterial species, sub-dominant in the original microbial community  
130 (Fig. S3). Their relative percentage abundance is shown in Fig. 1A. As an overall trend,  
131 *Geoalkalibacter subterraneus* (henceforth *Glk. subterraneus*) and a microorganism within the  
132 family *Geobacteraceae* were selected with a decrease of the abundance of *Clostridiaceae*  
133 species along the enrichment steps. The selection of *Glk. subterraneus* is not surprising since  
134 it is a well-known ARB (Carmona-Martínez et al., 2013; Miceli et al., 2012) and DMRB  
135 (Greene et al., 2009). This is in agreement with the expected effect of the DMRB-enrichment  
136 strategy. Moreover, *Glk. subterraneus* rather than other ARB was likely selected due to the  
137 saline conditions employed.

138

## 139 **3.2 ARB-enrichment in anodic biofilms**

### 140 **3.2.1 Maximum electron transfer rate ( $V_{\max}$ )**

141  $V_{\max}$  represents the maximum rate of electrons transfer from the biofilm to the anode  
142 per unit of time as described in the Supplementary Material. It is worth to notice that the  
143 parameter  $V_{\max}$  is estimated from the Gompertz model for the first time to describe the  
144 electrochemical performance of anodic biofilms. Therefore, no comparison is possible for this  
145 parameter with previous studies. It can be clearly seen that  $V_{\max}$  increased along the ARB-  
146 enrichment steps up to 300 C/d in B2 (Fig. 1B). However, for biofilm B3 formed from the  
147 DMRB-enrichment E3,  $V_{\max}$  significantly decreased to 150 C/d. This was likely due to the  
148 selection of less number of ARB or non-ARB at all after several enrichment steps as indicated  
149 in Fig. 1. Such non-ARB might have outcompeted ARB within the biofilm for substrate

150 consumption. The lower  $V_{\max}$  observed for E3 compared to E2 might also be due to a dilution  
151 of ARB present in the sediment throughout the DMRB-enrichment steps.

### 152 **3.2.2 Coulombic efficiency (CE) as an indicator of overall performances**

153 The fraction of electrons available in the substrate that ends up as electrical current in  
154 the system is denominated as Coulombic efficiency (CE) (Sleutels et al., 2011). CE is an  
155 indication of the actual amount of coulombs harvested from substrate oxidation and  
156 transferred by the biofilm to the anode. The increase of CE is one of the most important  
157 challenges in BESs, since most of the available electrons contained in the organic substrate  
158 are expected to be transferred to the anode to create an economically feasible process. In our  
159 study, CE gradually increased from  $30\pm 4\%$  (B0) until it reached a plateau at  $\geq 100\%$  (B1-  
160 B3). It is worth noticing that a single step of DMRB-enrichment and the use of its biomass as  
161 inoculum for B1 was enough to achieve a high CE. CE values  $>100\%$  were very likely due to  
162 the oxidation of  $H_2$  produced in the system at the cathode since a significant percentage of  $H_2$   
163 was detected in the gas phase at the maximum of current production ( $CO_2$  8.4 %,  $H_2$  42.12 %,   
164  $N_2$  33.26 % and  $CH_4$  11.30 %). The overestimation of the electrons recovered at the anode  
165 was probably due to  $H_2$  recycling, a well-known phenomenon occurring in single chamber  
166 BES (Lee & Rittmann, 2010). The evolution of  $H_2$  at the cathode in membrane-less BES can  
167 thus negatively affect the evaluation of the BES's performances in terms of CE due to the  
168 presence of  $H_2$ -oxidizing ARB able to convert  $H_2$  into electrons. Such conversion clearly  
169 produces a flow of electrons that not necessarily comes from the substrate fed to the BES  
170 which in turn increases CE values.

### 171 **3.2.3 Average maximum current density**

172 Although no increase in maximal current density ( $j_{\max}$ ) was observed after the first  
173 ARB-enrichment step (the highest  $j_{\max}$  was obtained for B0:  $4.5 A/m^2$ ), CE increased through  
174 enrichment steps from B0 to B2 as discussed above. The lack of gradual improvement of  $j_{\max}$



175 might suggest the presence of essential nutrients or perhaps mediators in the sediments that  
176 were required to achieve higher  $j_{max}$  values. The removal of such nutrients could have  
177 hindered further improvement of  $j_{max}$  in B1, B2 and B3. Moreover, a diluting effect of  
178 essential requirements for proper biofilm growth or electron mediators might have occurred  
179 along the DMRB-enrichment step chain. There are similarities between this phenomenon  
180 shown with successive iron-reduced enrichment cultures and previous studies where current  
181 densities had not been recovered among successive batch cycles of the same biofilm. Kim et  
182 al. (2005) tried a similar approach to improve the performance of electroactive biofilms by  
183 inoculating electrodes with biomass previously exposed to an iron-containing medium.  
184 Interestingly, they did not observe either significant performance improvement. The latest  
185 indicates that not all the DMRB can behave as ARB for the formation of electroactive  
186 biofilms, a finding only demonstrated for a few ARB (Richter et al., 2007). Although such  
187 diluting effect is not extensively reported in the literature, we suggest that a dilution of ARB  
188 might have caused the loss of activity in the formation of an electroactive biofilm.

### 189 **3.2.4 Microbial community analysis of anodic biofilms**

#### 190 **3.2.4.1 High microbial selection in ARB-enrichments caused by previous DMRB-** 191 **enrichments**

192 Interestingly, the CE-SSCP profiles obtained for ARB-biofilms were composed of  
193 only 1 or 2 dominant species (Fig. S3 B0-B3). This finding is well in agreement with previous  
194 studies in which BES inoculated with sediments presented a high anodic microbial  
195 simplification (Pierra et al., 2015). On the contrary, several studies report a highly diverse  
196 microbial community with bacteria (ARB and non-ARB), especially for BES inoculated with  
197 non-saline sources such as domestic wastewater. The high selection observed could be the  
198 result of specific experimental conditions such as pH 7.0, temperature at 37°C, synthetic  
199 medium composition, the fixed potential, and mostly the saline conditions applied.

#### 200 **3.2.4.2 *Geoalkalibacter* vs. *Geobacteraceae* in ARB-enrichments**

201 In B0 (Fig. 1C) the most dominant microorganisms colonizing the anodes were  
202 affiliated to *Desulfuromonas* spp. and *Glk. subterraneus* (ARB within the  $\delta$ -*Proteobacteria*  
203 class). From B1 to B3 *Glk. subterraneus* was selected as the dominant bacteria. This finding  
204 is consistent with previous reports where a majority of  $\delta$ -*Proteobacteria* was found in  
205 electroactive biofilms inoculated with sediments ( Miceli et al., 2012, Pierra et al., 2015, refer  
206 to supplementary materials). In this study, the selection of *Glk. subterraneus* from the  
207 DMRB-enrichment E1 permitted to increase the performances of the ARB-enrichment B1  
208 when compared to the biofilm directly developed from the initial sediment (B0) (Fig. 1).  
209 Besides, it is shown here that only one DMRB-enrichment step is required to select *Glk.*  
210 *subterraneus* under saline conditions. Indeed, the use of the first DMRB-enrichment as  
211 inoculum (E1) provided both an ARB enriched community B1 and a DMRB community (E2).  
212 The most abundant bacterium in B1 was 99% similar to *Glk. subterraneus* at 16S rDNA  
213 sequence level and the most abundant bacterium in E2 was an unknown species belonging to  
214 the *Geobacteraceae* family with 89% similarity to *Glk subterraneus*. This bacterium  
215 belonging to *Geobacteraceae* family presented abilities to reduce Fe(III) as many  
216 *Geobacteraceae* do. Nevertheless, it has probably a low capacity to transfer this property to  
217 graphite anode respiration, as previously described for other DMRB (Richter et al, 2007).  
218 Therefore, the emergence of this *Geobacteraceae* in the detriment of *Glk. subterraneus* can be  
219 explained due to the decrease of electroactive efficiency of biofilms after the first enrichment  
220 step.

#### 221 **3.2.4.3 Convergence of bacterial communities correlates with electroactive** 222 **performances**

223 Fig. 2 presents the results of a principal component analysis (PCA) showing the  
224 differences in the microbial community structures of the bacterial communities during the

225 successive DMRB- and ARB-enrichments. CE-SSCP profiles are compared by using the  
226 Euclidian distances between CE-SSCP patterns as detailed elsewhere (Pierra et al., 2014).  
227 According to the PCA analysis, the closer the points from each other, the more similar the  
228 microbial communities are. Both DMRB-enrichment cultures and ARB-enriched biofilms in  
229 comparison to the sediment are presented. A positive correlation between the optimum  
230 performance (Fig. 1) and the similarity between DMRB-reducing and ARB-reducing  
231 microbial communities was observed (Fig. 2). The best performing ARB-enrichment (B2) (in  
232 terms of electrons recovered as CE calculated) and its corresponding DMRB-enrichment (E2)  
233 were closely related.

234 One unanticipated finding was a divergence in the microbial community composition  
235 between DMRB- and ARB-enrichments especially between E3 and B3. This might suggest a  
236 difference between extracellular electron transfer mechanisms between DMRB- and ARB-  
237 enrichments. Mechanisms employed by DMRB and ARB probably required slightly different  
238 physiologies. Thus, the selection of bacteria on DMRB-enrichments surely conducted the  
239 emergence of a specific consortium specialized on Fe(III) respiration, not necessarily able to  
240 use an electrode material as final electron acceptor, as previously observed by Richter et al.  
241 (2007). Nevertheless, here one step of ARB-enrichment (B0) was enough to achieve  
242 successful substrate conversion in terms of CE (see Fig. 1).

243 -Please insert **Figure 2** here-

#### 244 **4 Conclusion**

245 This study reported the effect of employing a previously grown culture enriched in iron-  
246 reducing microorganisms (DMRB-enrichment) as inoculum to develop electroactive biofilms  
247 (ARB-enrichment). One single step of DMRB-enrichment was enough to favor the selection  
248 of the ARB *Glk. subterraneus*. This recently characterized ARB provided interesting  
249 electroactive skills considering the saline conditions used. Then, the divergence in microbial

250 community composition, associated with a decrease of electroactive efficiency, suggests that  
251 respiration on Fe(III) and on anode presumably require dissimilar skills.

## 252 **5 Acknowledgements**

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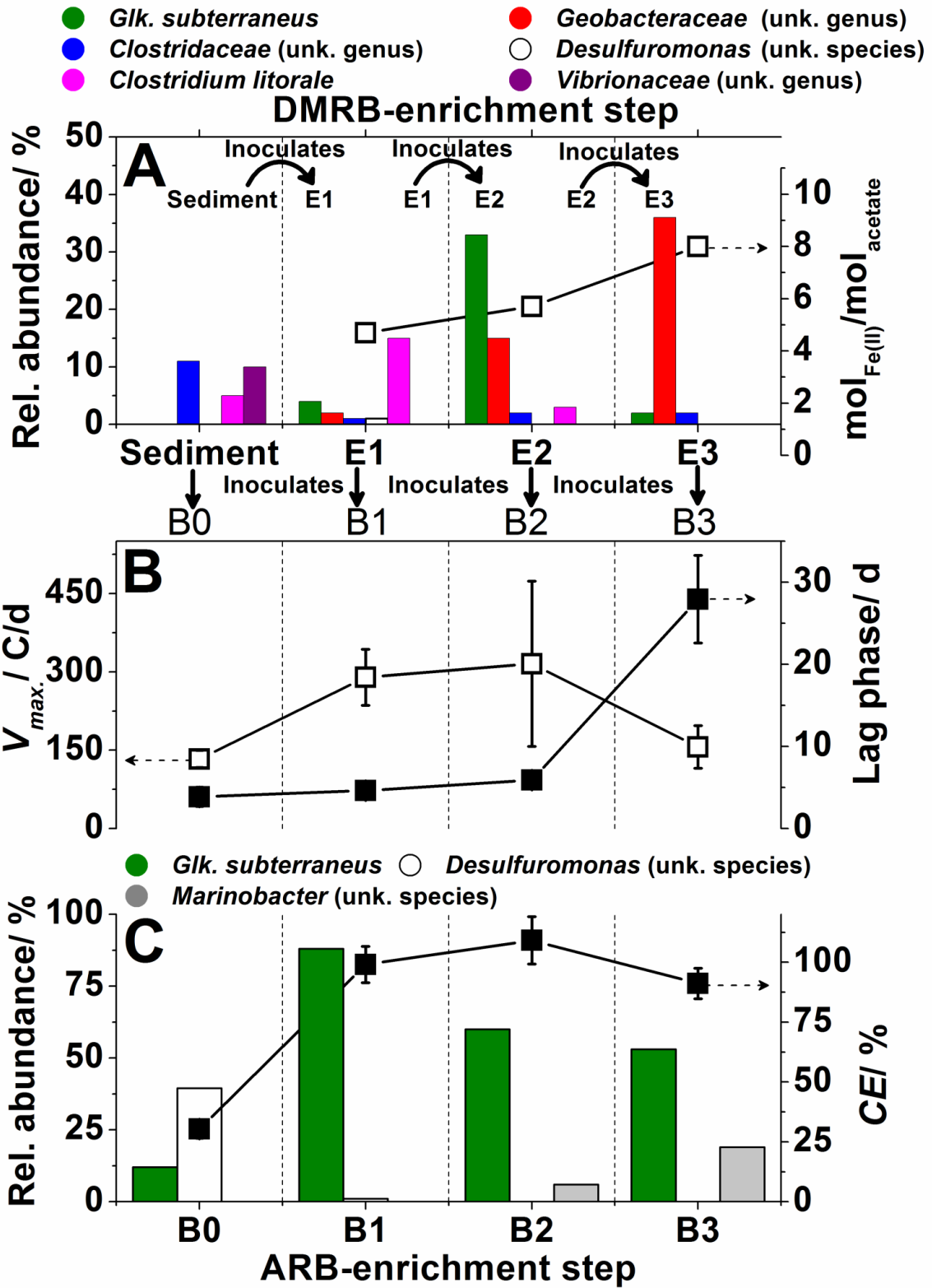
## 301 **Figure captions**

302 **Fig. 1** - A) Relative abundance of main bacteria in the Sediment used as inoculum and  
303 DMRB-enrichments E1, E2 and E3 (columns). Conversion rate of acetate into Fe(II) in  
304 DMRB-enrichments (empty squares). B) Electron transfer dynamics for ARB-enrichments.  
305 Maximum charge production rate (empty squares) and Lag phase (full squares). C) Relative  
306 abundance of main bacteria (columns) and Coulombic efficiencies (CE in %) (full squares) .  
307 Note: Rel. abundance values are reported for those ARB found at more than 9 % in at least 1  
308 bacterial community.

309 **Fig. 2** - Principal Component Analysis (PCA) biplot of CE-SSCP profiles. For each biofilm  
310 (B0, B1, B2, B3), an average of 4 replicate profiles is presented ( $\diamond$ ). CE-SSCP profiles of the  
311 sediment originate inoculum E0 ( $\bullet$ ) and of iron-enrichments microbial communities (E1, E2  
312 E3) are also presented ( $\circ$ ). The first two principal components (Axis1 and Axis2) explained  
313 61.1% of the genetic variation.

314

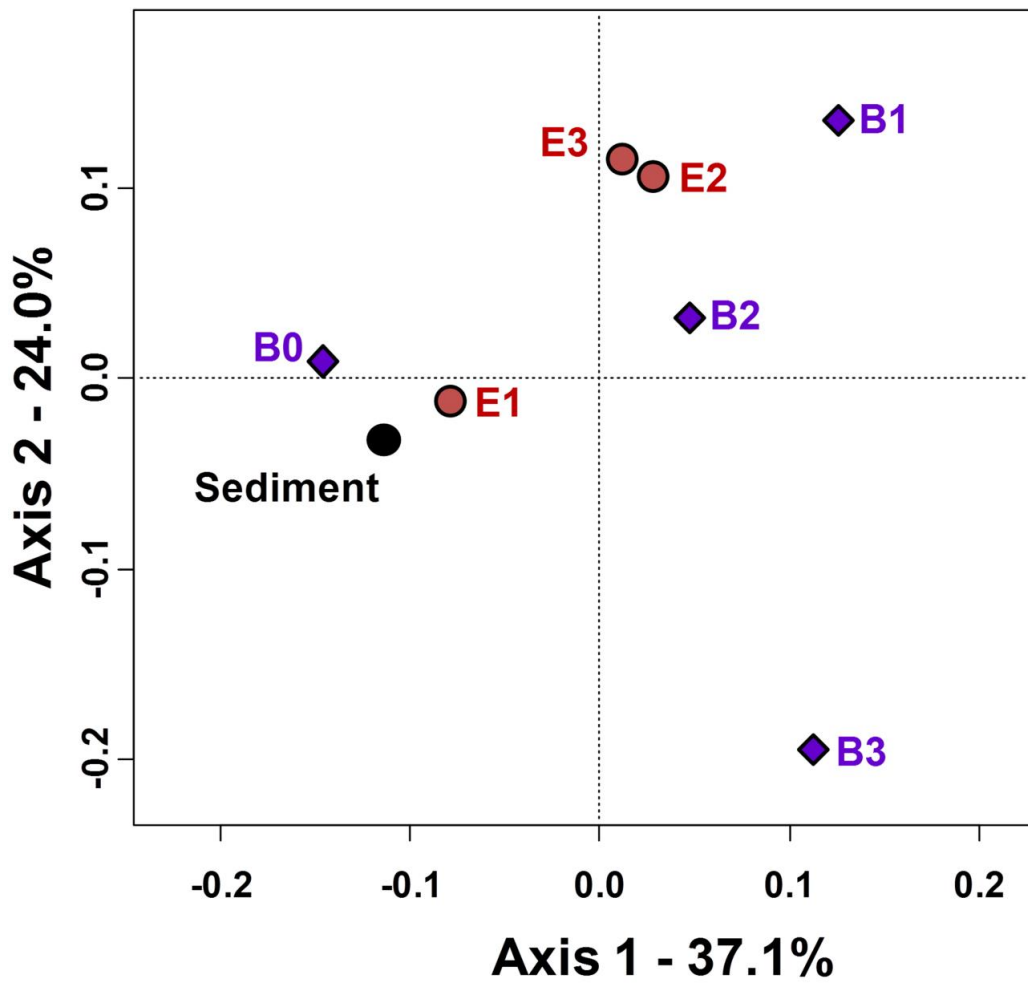
315 Fig. 1:



316

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318 Fig. 2 :



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