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1 Development of bioanodes rich in exoelectrogenic bacteria using iron-
2 rich palaeomarine sediment inoculum

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8

9

10 **Abstract**

11 Microbial Fuel Cells (MFC) convert energy stored in chemicals into electrical energy thanks to
12 exoelectrogenic microorganisms who also play a crucial role in geochemical cycles in their
13 natural environment, including that of iron. In this study, we investigated paleomarine
14 sediments as inoculum for bioanode development in MFCs. These sediments were formed
15 under anoxic conditions ca. 113 million years ago and are rich in clay minerals, organic matter,
16 and iron. The marlstone inoculum was incubated in the anolyte of an MFC using acetate as
17 the added electron donor and ferricyanide as the electron acceptor in the catholyte. After
18 seven weeks of incubation, the current density increased to 0.15 mA.cm⁻² and a stable +700
19 mV open circuit potential was reached. Community analysis revealed the presence of two
20 exoelectrogenic bacterial genera, *Geovibrio* and *Geobacter*. Development of electroactive
21 biofilms was correlated to bulk chemical transformations of the sediment inoculum with an
22 increase in the Fe(II) to Fe_{total} ratio. Comparisons to sediments sterilized prior to inoculation
23 confirmed that bioanode development derives from the native microbiota of these
24 paleomarine sediments. This study illustrates the feasibility of developing exoelectrogenic
25 biofilms from iron-rich marlstone and has implications for the role of such bacteria in broader
26 paleoenvironmental phenomena.

27

28 **Keywords:**

29 Exoelectrogenic bacteria, biofilms, bio-anode, paleomarine sediments, clay minerals

30 **Introduction**

31 The process of Fe (III) reduction by exoelectrogenic bacteria is crucial in the iron geochemistry
32 cycle and plays a vital role in the degradation of natural and contaminant/waste organic
33 compounds [1–3]. Exoelectrogenic bacteria have potential applications in the fields of
34 bioremediation and biotechnology, as they can oxidize organic contaminants such as aromatic
35 hydrocarbons anaerobically in anoxic petroleum-contaminated aquifers and simultaneously
36 reduce Fe (III) [4]. For example, one study suggests that exoelectrogenic bacteria also play a
37 fundamental role in Precambrian banded iron formations, as evidenced by the strong
38 correlation between isotopically-light carbonates and iron minerals accumulations that
39 occurred through a combination of organic matter mineralization and Fe (III) reduction [5].
40 Thus, much research has been conducted to better understand the interaction between
41 microorganisms and sediments in different environments, as this relationship plays a role in
42 biogeochemical cycles where bacteria can alter the physical and chemical state of sediments
43 [6]. Marine sediments are well-known as a source of abundant electrochemically active
44 bacteria [7] and have been used as inoculum in sediment Microbial Fuel Cells (MFCs) for
45 simultaneous energy production and bioremediation [8]. It is essential to highlight that the
46 amount of energy obtained in the MFCs devices depends on several factors, including the
47 quantity of organic matter and type of exoelectrogenic microorganisms present in the
48 sediments [9,10]. Moreover, it can be argued more broadly that climatological factors and
49 geological conditions play a role in MFC development, by influencing the microbiota
50 composition of natural inoculum. In this context, the exploration of paleomarine sediments
51 from a geological perspective offers a promising avenue for pinpointing actual and past
52 environments conducive to the development of microorganisms, and selecting appropriate
53 sediments for inoculation of MFCs [11].

54 In this study, we focus on the development of electroactive biofilms using paleomarine
55 sediment inoculum formed in hemipelagic environment, characterized by a large deposition
56 of organic-rich layers with accumulation of weathering products [12]. These ancient geological
57 deposits are rich in clay minerals, including smectite, illite, kaolinite and chlorite, but also
58 showcase high iron contents (primarily Fe(III)) as well as organic matter. These conditions may
59 provide a favorable environment for the development of electroactive bacteria. Herein we
60 demonstrate the development of acetate-metabolizing bioanodes in a microbial fuel cell
61 system using these iron-rich paleomarine sediments as the inoculum. Physico-chemical

62 characterization of the sediments in the anolyte after continuous operation shows a distinct
63 increase in the proportion of Fe(II) oxides to the total iron oxide content (Fe(II)/Fe_{total})
64 consistent with the presence of exo-electrogens utilizing abundant Fe(III) in the sediments as
65 the sole electron acceptor under anaerobic conditions. Using 16S rRNA amplicon sequencing
66 we correlate these changes in sediment composition to the development of mixed biofilms
67 containing *Pelobacter* genus and some well-known exo-electrogens species from the genera
68 *Geovibrio* and *Geobacter*.

69 **2. Materials and methods**

70 **2.1 Sample collection**

71 The Paleomarine Sedimentary rock (PS) used in this study are marlstone (mixture between
72 clay and calcite), with organic matter content around 1.3 %. The samples were collected from
73 Col de Pré-Guittard section of the Vocontian basin in France, located approximately at 11 km
74 north-northwest of Rémuzat, Drôme (Supporting Information Figure S1). The clay mineral
75 assemblages of the Col de Pré-Guittard section consist mainly of smectite (averaging 63%),
76 illite (averaging 19%), and kaolinite (averaging 11%), along with approximately 5% chlorite
77 [13]. The selected marlstone sample was crushed into powder using an agate mortar to
78 achieve an average particle size < 125 µm. X-ray diffractometry (XRD) was employed to
79 identify the mineral phases present in the sediment, revealing the presence of quartz, calcite,
80 gypsum, and clay minerals (Supporting Information Figure S2). The Scanning electron
81 microscopy (SEM) image of the PS revealed a platy-shaped structure of clay mineral particles.
82 The elemental composition of PS recorded the presence of high levels of alumino-silicate
83 (approximately 20% of Si and 8% of Al) arising from the clay, as well as a significant quantity
84 of Fe element, approximately 5.8% (Supporting Information Figure S2).

85 **2.2 Chemical reagents**

86 Potassium acetate (> 99%, anhydrous), glutaraldehyde (25% solution), potassium
87 hexacyanoferrate III (K₃Fe(CN)₆), potassium phosphate dibasic (K₂HPO₄), and potassium
88 phosphate monobasic (KH₂PO₄) were purchased from Sigma Aldrich and used without further
89 purification.

90 **2.3 Bio-anode Setup & electrochemical measurements**

91 The marlstone samples were incubated under anoxic conditions that are favorable for the
92 development of exoelectrogenic bacteria as previously reported [14] [15]. Briefly, an H-type
93 reactor was used with 250 mL compartments separated by a Cation Exchange Membrane

94 (Fumasep® FKM). Graphite rods (Morgan AM&T) with a diameter of 0.5 cm and height of 20
95 cm were used as electrodes in both compartments. For the anodic compartment, the
96 electrode was cleaned using pure water in ultrasound for 10 min, then covered by parafilm to
97 fix the surface at 4.9 cm². In the anodic compartment, 8.05 g of marlstone samples was mixed
98 with 250 mL of phosphate buffer (10 mM) prepared using ultrapure (18.2 MΩ.cm) water. The
99 chosen concentration is consistent with the previous experiments using low ionic strength
100 electrolytes which successfully developed bioanodes from mixed inoculum[16] The pH of the
101 anodic compartment was measured and adjusted to pH 7 using 2M NaOH. Potassium acetate
102 (10 mM) was regularly added as an electron donor from filtered anoxic stock solutions.
103 The cathodic compartment was filled with 250 mL of phosphate buffer (10 mM) containing
104 potassium hexacyanoferrate (50 mM) as an electron acceptor. The cathode showed a stable
105 potential (*ca.* 0.2 V vs Ag/AgCl 3 M KCl) which remained constant throughout the experiment.
106 The two compartments were connected by an external resistor 1-4 kΩ to match the internal
107 resistance of the MFC as determined via impedance spectroscopy measurements using a Multi
108 Autolab / M204 potentiostat [15]. These pilots are designated as PS/MFC (PS/MFC 1 and
109 PS/MFC 2).
110 A control cell (PS/Control) was also established and incubated under identical conditions
111 without connection to an external cathode designated as (PS/Ctrl 1 and PS/Ctrl 2). To maintain
112 the MFC under anaerobic conditions, the anodic compartment was purged with argon gas for
113 30 minutes once every 5 days on average. MFCs were incubated in the dark at 30 °C in a
114 thermostatic bath (Supporting Information Figure S3). The open circuit potential (OCP)
115 between the electrodes was measured periodically using a digital multimeter or potentiostat.
116 MFCs were left at open circuit for at least 30 minutes prior to taking the measurement. To
117 monitor the development of exo-electrogenic bacteria from the marlstone samples, the bio-
118 anode was regularly studied using cyclic voltammetry in the range -0.8 to +0.4 V vs Ag/AgCl 3
119 M KCl at a scan rate of 2 mV/s unless otherwise specified. Electrochemical measurements
120 were performed using a three-electrode set-up where the bioanode served as the working
121 electrode, Ag/AgCl (3 M KCl) as the reference electrode placed in the anolyte, and the cathode
122 (graphite rod) was used as a counter electrode. All experiments were conducted in duplicate
123 under an inert atmosphere using Ar saturation. Samples of solution or sediments from the
124 MFCs were taken under argon flux with sterile syringes to minimize disturbance to the
125 bioanodes and preserve anaerobic conditions. Additional control experiments were

126 performed using sterilized inoculum: the paleomarine sediments were dispersed in a small
127 volume of ultrapure water and autoclaved (Autoclave Steril Elite™, Fisher Scientific brand) for
128 1 hour at 121°C and then incubated under identical conditions to the PS/MFC.

129

130 **2.4. X-Ray Photoelectron Spectroscopy (XPS)**

131 In all cases, powder samples for XPS analysis were prepared by pressing the powder (0.1 g) on
132 carbon tape mounted on the conductive sample holder. XPS data were obtained using a
133 NEXSA G2 (ThermoFischer Scientific) spectrometer using an Al K α X-ray source working at
134 1486.6 eV with a spot size of 200 μm^2 . Survey spectra (0-1000 eV) were acquired with an
135 analyzer pass energy of 200 eV (1 eV/step); high-resolution spectra used a pass energy of 50
136 eV (0.1 eV/step). Charge compensation using a flood gun was applied to all powder
137 preparations and the binding energies corrected to the position of the C 1s peak assigned to
138 adventitious carbon at 284.8 eV. Spectra were peak-fitted using commercial software
139 (CasaXPS Version 2.3.18). Deconvolutions were performed using Shirley background
140 subtraction in all cases. Peak areas used in quantitative analysis were normalized by relative
141 sensitivity factors for atomic percentage determination (C 1s = 1, O 1s = 2.93, Si 2p = 0.817, Ca
142 2p = 5.07, Fe 2p = 16.42, K 2p = 3.97, Mg 2s = 0.575, P 2p = 0.817, Na 1s = 8.52, Al 2p = 0.537).

143 **2.5. X-Ray Diffraction (XRD)**

144 To determine the mineral phases, present in the marlstone samples, X-Ray Diffraction (XRD)
145 was conducted. Dry powder samples were analyzed using a Bruker D8 Endeavour
146 diffractometer equipped with CuK α radiations, a LynxEye_XE_T detector, and a Ni filter. The
147 instrument was set at a voltage of 40 kV and an intensity of 25 mA. The goniometer scanning
148 ranged from 2° to 65° for each analysis. The identification of mineral phases was accomplished
149 by analyzing their primary diffraction peak (d001).

150 **2.6. Scanning Electron Microscopy imaging (SEM) and Inductively Coupled Plasma Mass 151 Spectroscopy (ICP-MS)**

152 SEM samples were prepared following the protocol of reference [17]. First, the samples are
153 fixed overnight with 2.5% glutaraldehyde in buffer phosphate solution (0.1 M, pH 7) at room
154 temperature. Then, samples were washed using a phosphate buffer solution (pH 7) and
155 immersed respectively in an aqueous solution containing increasing ethanol concentrations
156 from 50%-100% as previously reported [15]. Finally, the samples were critical-point dried to

157 replace the remaining water and ethanol solvent with CO₂ and Au-coated. The analysis was
158 performed using a JEOL JSM 7100 F EDS EBSD Oxford Scanning Electron Microscope.
159 To estimate the overall concentration of oxides and total iron content, we used an ICP-MS
160 (Inductively Coupled Plasma-Mass Spectroscopy) iCapQ ThermoFisher performed by the
161 National Service of Rocks and Minerals Analysis (SARM: [http://helium.cprg.cnrs-
162 nancy.fr/SARM/](http://helium.cprg.cnrs-nancy.fr/SARM/)) in Nancy. Prior to the analysis, it is necessary to prepare the samples through
163 alkaline fusions using lithium metaborate (LiBO₂). Fe(II) content was established using
164 potentiometric titration for calculations of the ratio Fe(II)/Fe_{total}.
165

166 **2.7. DNA extraction from the biofilm**

167 After the incubation and development of the biofilm, DNA extraction was performed on all
168 samples using the DNeasy PowerSoil DNA extraction kit (Qiagen®) according to the
169 manufacturer's protocol. Prior to DNA extraction the biofilm developed on the surface of the
170 electrode was gently scraped using a spatula sterilized with absolute ethanol. To investigate
171 the distribution of bacterial communities throughout the entire anodic compartment, DNA
172 was also extracted from the solution-sediment interface. The concentration of DNA was
173 verified using a Nanodrop® and all samples reported had sufficient DNA to proceed with
174 sequencing. The 16S rRNA gene sequencing was done by the GeT-Biopuces laboratory at INSA
175 Toulouse. The variable region V4-V5 of the 16S rRNA was targeted using the primers 515F (5'-
176 GTGCCAGCMGCCGCGGTAA-3') and 928R (5'-CCCCGYCAATTCMTTTRAGT-3'). The amplified
177 products were then sequenced using an Illumina MiSeq instrument, and the results were
178 obtained in the FastQ format [15].

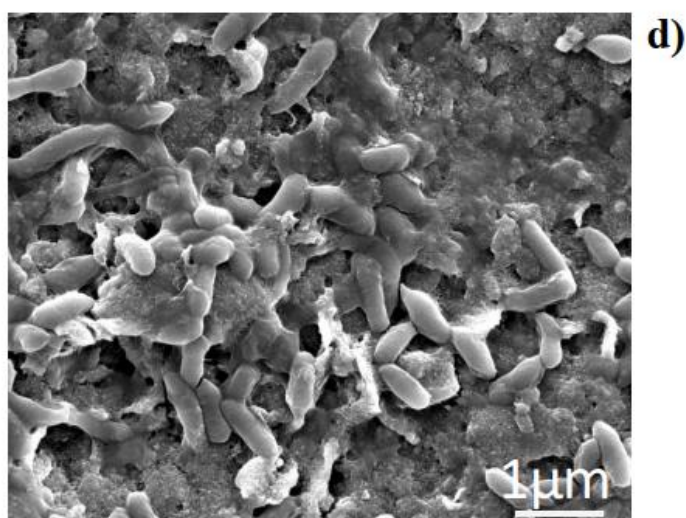
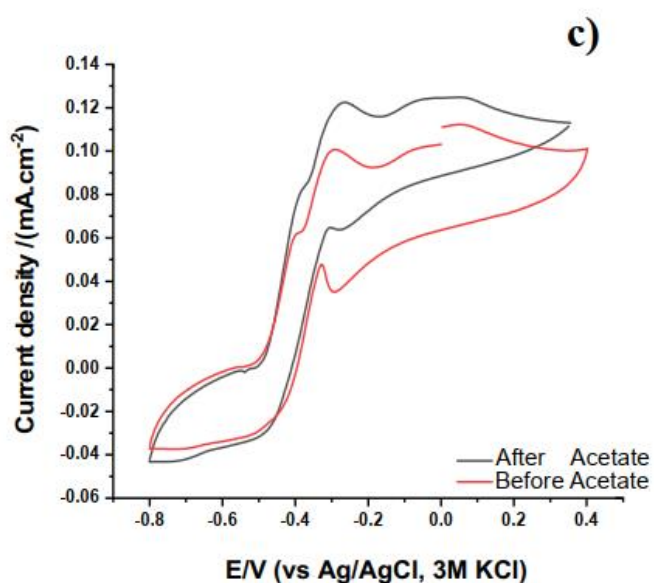
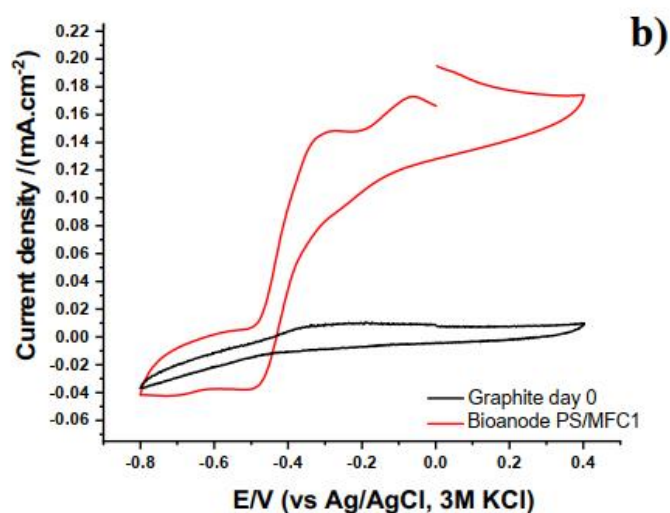
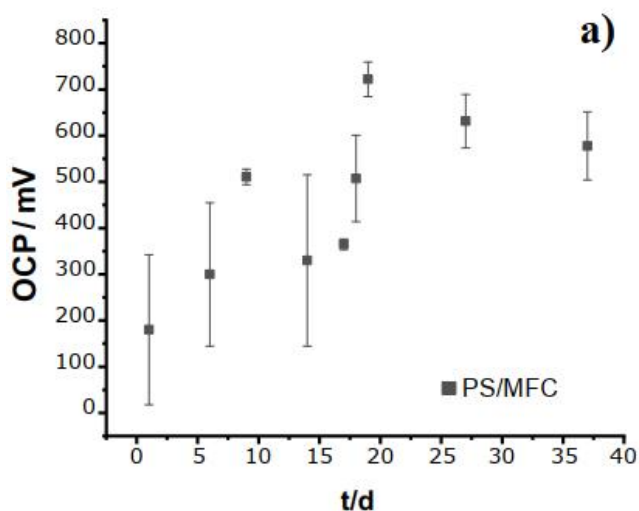
179 **3. Results**

180 **3.1. Characterization of the bioanode developed at the surface of the electrode**

181 During the initial days of incubation, the Open Circuit Potential (OCP) was 200 mV (Figure 1a)
182 versus the cathode which held a constant potential of 0.2 V vs Ag/AgCl (3 M KCl) throughout
183 the experiment, maintained using 50mM of potassium hexacyanoferrate (III) as an electrolyte
184 in the cathodic compartment [18]. Cyclic voltammograms showed a typical graphite
185 background with only capacitive current (Figure 1b, black curve). After 14 days of incubation,
186 the OCP of PS/MFC pilots significantly increased up to a maximum of *ca.* 700 mV and remained
187 stable upon periodic measurement thereafter. This OCP increase is attributed to the
188 development of an electroactive biofilm capable of acetate oxidation at the surface of the
189 graphite rod. This is confirmed in cyclic voltammetry studies of the mature bioanodes (Figure
190 1b, red curve) developed in PS/MFC1 which shows a sigmoidal oxidation curve with onset
191 between -0.6 and -0.5 V vs Ag/AgCl 3 M KCl attributed to catalytic acetate oxidation. Additional
192 redox peaks centered between -0.42 to -0.30 V are attributed to outer membrane
193 cytochromes present in the electroactive biofilm [19], [15]. A further oxidation peak was
194 observed between -0.2 V and -0.18 V vs Ag/AgCl 3 M KCl consistent with the presence of iron
195 oxides surrounding the biofilm as previously reported [15].

196

197



198

199 **Figure 1.** a) Open circuit potential (OCP) of both PS/MFC Pilots (MFC1 and MFC2) obtained after 40
 200 incubation days. b) Cyclic voltammogram recorded from the PS/MFC1 pilot from the first day of
 201 incubation (black curve) and again after 90 days of incubation (red curve). c) Cyclic voltammograms of
 202 bioanode PS/MFC1 before and after the addition of 10 mM acetate. d) SEM image of a mature mixed
 203 bioanode developed from PS/MFC1.

204 At this point, such biofilms yielded reproducible voltammograms with the sigmoidal catalytic
205 profile, in both pilots (PS/MFC1 and PS/MFC2; see supporting information Figure S4b)
206 compared to the sterilized pilots that show no catalytic profile (see supporting information
207 Figures S5, S6b) with an OCP around 400 mV compared to the PS/MFC that recorded 700 mV
208 at the same period of incubation (see supporting information Figure S6a). The specificity of
209 the acetate response was confirmed by an enhancement in the catalytic current upon re-
210 addition of 10 mM acetate after 24h of constant turnover in PS/MFC1 (Figure 1c). The acetate
211 dependence was also replicated on other independent pilots (see supporting information
212 Figure S4a). These results suggest that the exoelectrogenic bacteria developed at the
213 bioanode in PS/MFC pilots provided from the initial paleomarine inoculum. Moreover, a
214 complete loss of catalytic activity was observed after the removal of the biofilm for DNA
215 extraction (Supporting Information Figure S7), thus confirming that the catalytic response
216 observed could be attributed to a biofilm developed on the electrode surface (see supporting
217 information Figure S8). SEM imaging of the bioanode after glutaraldehyde fixation confirmed
218 the presence of a mixed bacterial biofilm at the electrode surface. A representative image is
219 shown in Figure 1d and indicates that bacteria grow across the graphite surface (Supporting
220 Information Figure S9a). The control electrode which was not electrically coupled to a cathode
221 through a resistor also shows significant biomass but not a continuous biofilm as same form
222 as anodes operated as MFCs (Supporting Information Figure S9b-d and S10). This result is
223 consistent with community analysis from 16S as discussed below.

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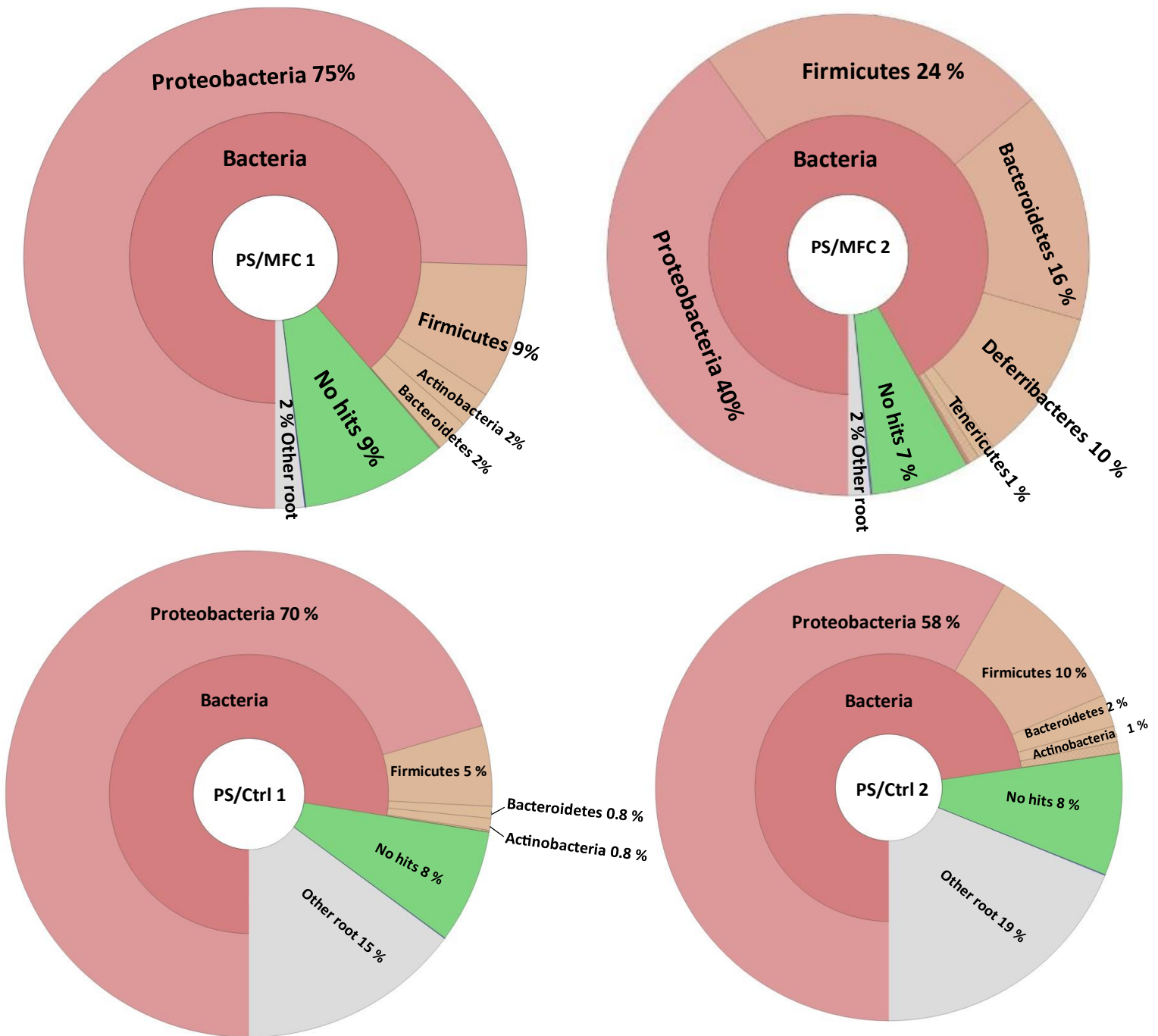
225 3.2. Extraction of the biofilm and community determination

226 Based on electrochemical experiments and SEM images, it is evident that a mixed community
227 electroactive biofilm has developed on the surface of the graphite electrode. To enhance
228 electron transfer efficiency and gain a better understanding of the electron transfer
229 mechanism, it is crucial to identify and study the physiology of anodic microbial communities
230 developed on the electrode surface, as this serves as a fundamental aspect in optimizing
231 electron transfer processes and improving overall system performance [7].

232 To determine the nature of the biofilm colonizing the bioanode, 16S amplicon sequencing
233 analysis was conducted on the PS/MFC pilots, SPS/MFC pilots, controls, and PS day 0. The
234 sequencing analysis for all the pilots revealed the abundance of three phyla Proteobacteria,
235 Firmicutes, and Bacteroidetes (Figure 2). These findings align with the initial composition of
236 the marlstone samples before incubation (see Supporting Information Figure S11). Notably,
237 we recorded the growth of *Deferribacteres* phylum at the PS/MFC (PS/MFC 2) with a relative
238 abundance of around 10 %. This phylum was initially present in the marlstone samples prior
239 to incubation with a low abundance of less than 0.1% and developed under the incubation
240 conditions to reach 10%. The growth of this phylum suggests the development of
241 exoelectrogenic bacteria at the anodic compartment such as those of the *Geovibrio* genus,
242 capable of reducing metal ions. This genus has been found to have industrial applications,
243 including the conversion of organic compounds to biofuels [20].

244 These results are not surprising, knowing that the Proteobacteria are the most abundant
245 phylum in soil [21], which includes important exoelectrogenic bacteria such as those of the
246 *Geobacter* genus, able to oxidize organic compounds completely to carbon dioxide with an
247 anode serving as the sole electron acceptor [22] [23]. Likewise, in the Firmicutes phylum, some
248 isolated members can transfer electrons to solid phases as electron acceptors [24], including
249 iron and manganese, as part of a respiratory mechanism [25]. Bacteroidetes phylum has been
250 associated with the anodic biofilm and reported as a fermentative phylum able to hydrolyze
251 complex organics molecules like cellulose[26].

252



254 **Figure 2:** Relative abundance of bacterial communities (at the Phylum level) colonizing the electrode
 255 from PS/MFC bioanodes (PS/MFC1 and PS/MFC 2) and from PS/Control (PS/Ctrl 1 and PS/Ctrl 2).

256

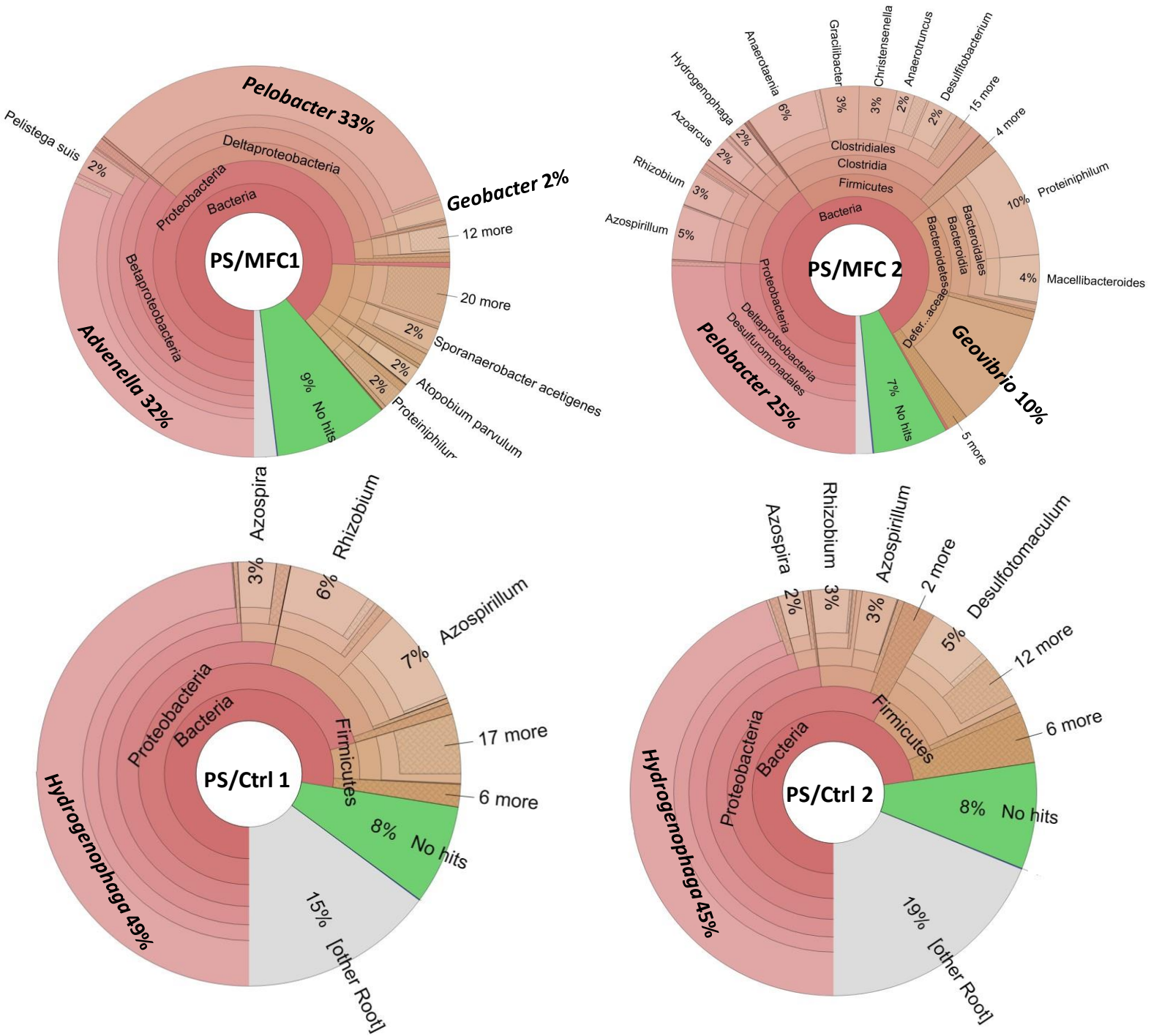
257 In more detailed sequencing data, we recorded the abundance of the *Pelobacter* genus in both
258 PS/MFC duplicates, constituting approximately 33% and 25% of the microbial community
259 recorded respectively in PS/MFC 1 and PS/MFC 2 (Figure 3). The abundance of *Pelobacter* at
260 the anode surface is not surprising as *Pelobacter* is often isolated from anodic biofilms [27]
261 including a recent study from our group [15]. In general, bacteria of the *Pelobacter* genus are
262 not able to oxidize acetate and perform extracellular electron transfer simultaneously. While
263 recent studies recorded the presence of some species of *Pelobacter* (*Pelobacter carbinolicus*)
264 able to reduce Fe(III) and S(0) by an incomplete oxidation of organic substrates[28], they
265 typically reduce smaller quantities of Fe(III) compared to *Geobacter* and *Desulfuromonas*
266 species [29]. Indeed, *Pelobacter carbinolicus* is not capable of direct electron transfer to
267 graphite anodes [30]. However, a recent publication in our team highlighted the presence of
268 unidentified species of *Pelobacter* able to colonize the electrode and apparently transfer their
269 electrons to the electrode directly [15]. Moreover, we registered the presence of two well-
270 known exoelectrogenic bacterial genera, *Geobacter*, and *Geovibrio* with relative abundances
271 of approximately 10% and 2% respectively, in PS/MFC 1 and PS/MFC 2. Previous studies have
272 documented the occurrence of these genera in the anodic compartment, as they possess the
273 ability to metabolize acetate and directly transfer electrons to insoluble electron acceptors
274 [25]. Considering the low abundance (less than 1%) of the three genera in the initial sediment,
275 we suggest that their development is favored through selective pressure under anaerobic
276 conditions in the presence of acetate. We therefore attribute the observed catalytic behavior
277 of PS/MFC pilots in acetate oxidation to the presence of a mixed biofilm rich in these known
278 electricigens developed from a natural paleomarine sediment inoculum.

279 Considering the sequencing analysis, the presence of well-known exoelectrogenic bacteria in
280 PS/MFC pilots is nonetheless relevant, as they may be implicated in anaerobic transformations
281 of the marlstone samples. Community analysis of PS/Control (PS/Ctrl1 and PS/Ctrl 2) pilots
282 incubated without external connection to a cathode showed radically different results to the
283 PS/MFC pilots (Figure 2). Surprisingly, we recorded a large predominance of *Hydrogenophaga*
284 in both PS/MFC duplicates, constituting approximately 49% and 45% of the microbial
285 community recorded respectively in PS/Ctrl 1 and PS/Ctrl 2. According to previous studies, the
286 *Hydrogenophaga* genus occurred often in the anodic biofilm community [31,32], developed
287 under anaerobic conditions with suitable electron acceptors as nitrate or electrode in a
288 Microbial Fuel Cell[32]. Moreover, *Hydrogenophaga* contains a species known as

289 *Hydrogenophaga electricum* that can develop under fixed flow of H₂ using a reactor connected
290 to an electrolyzer to produce a stable concentration of H₂ [33]. Based on our findings and
291 current understanding, the other bacteria observed in the PS/control samples do not possess
292 a known capability for producing hydrogen from acetate. This leads to the hypothesis that
293 hydrogen production may be attributed to unidentified bacteria or a symbiotic relationship
294 between different bacteria present in the environment. Further investigations are currently
295 underway to explore this possibility and shed more light on the mechanisms involved.

296 The low abundance of known exoelectrogenic bacteria at the electrode surface, can be
297 explained by the presence of another electron acceptor used by bacteria instead of the
298 electrode. To substantiate this hypothesis, we investigated the interface solution-sediment to
299 explore the presence of a possible exoelectrogenic bacteria at the sediment surface. The
300 sequencing results of the PS/MFC were surprising, indicating the abundance of *Advenella*
301 *genus* (around 81% in PS/MFC1) known for their potential to degrade alkanes and diesel [34],
302 and the development of *Azospirillum* (41% in PS/MFC 2) capable of nitrogen fixation [35]
303 (Supporting Information Figure S12). However, we noted the absence of *Pelobacter*,
304 *Geovibrio*, and *Geobacter* recorded previously at the surface of the bioanode, suggesting that
305 this genus preferentially colonizes the electrode (Figure 2). In contrast, the PS/Ctrl pilots
306 shows the abundance of the species *Hydrogenophaga* respectively around 40 % in PS/Ctrl 1
307 and 28% in PS/Ctrl 2 (Supporting Information Figure S13). Moreover, the sequencing data of
308 SPS/MFC pilots developed from the sterilized inoculum shows a mixed biofilm with the
309 absence of well-known exoelectrogenic bacteria (Supporting Information Figure S14).
310 Suggesting that the sterilization effectively eliminated the initial bacteria present in the
311 paleomarine sediment and we attribute the development of the exoelectrogenic bacteria to
312 the initial paleomarine inoculum.

313



314 **Figure 3:** Summary of community analysis (species levels) colonizing the PS/MFC bioanodes,
 315 PS/Control bioanodes
 316
 317

318 **3.3 Bulk Chemical Changes in Sediment Samples**

319 Under anaerobic conditions, the marlstone incubated in a microbial fuel cell (MFC) system,
320 using acetate as an electron donor and an electrode rod as an electron acceptor, promote the
321 growth of exoelectrogenic bacteria, as previously discussed. In addition, we observed notable
322 environmental changes marked by the appearance of black suspended aggregate (Supporting
323 Information Figure S15). The diffractogram analysis of the initial sediment from the marlstone
324 sample indicated the presence of various minerals such as gypsum (CaSO_4), calcite (CaCO_3),
325 quartz (SiO_2), and different clay minerals rich in iron like smectite, illite, kaolinite, and chlorite
326 [13].

327 The X-ray diffraction performed on the black powder recovered from the PS/MFC shows the
328 dissolution of the gypsum (CaSO_4 , diffraction peaks G in Figure 4). Likewise, the clay mineral
329 assemblage exhibited a broadening and shift in peak positions, indicating possible alterations
330 in lattice parameters and crystalline structure. Additionally, the distribution of Fe in layer
331 structure can affect the chemical reactivity of clay by affecting the layer charge which can
332 change the properties of minerals in terms of cation exchange capacity and swelling [36] [37].

333 The detection of new mineral phases is difficult in our study as we used impure natural
334 sediment without any previous treatment, which contains impurities and mixed elements
335 (Figure 4).

336

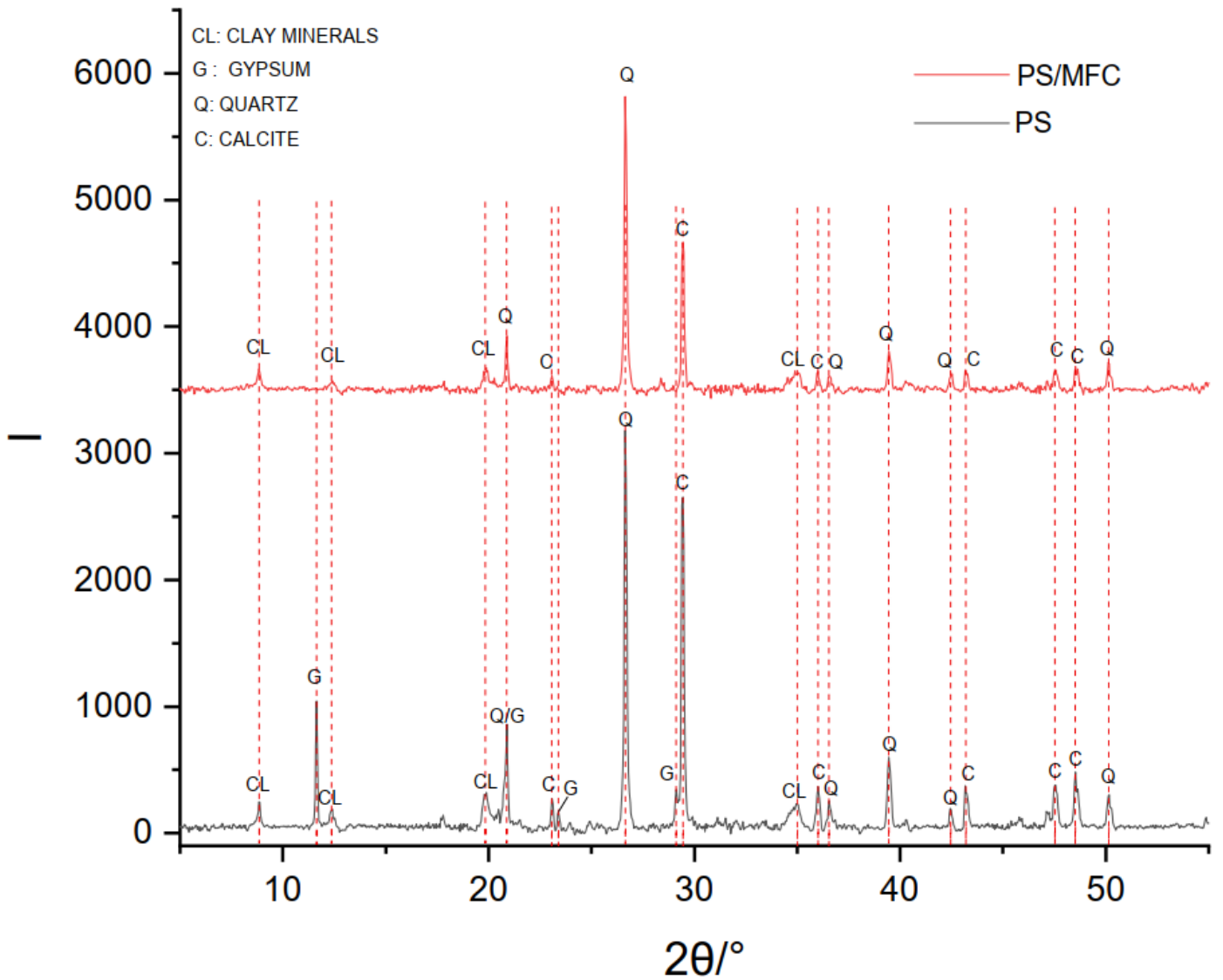
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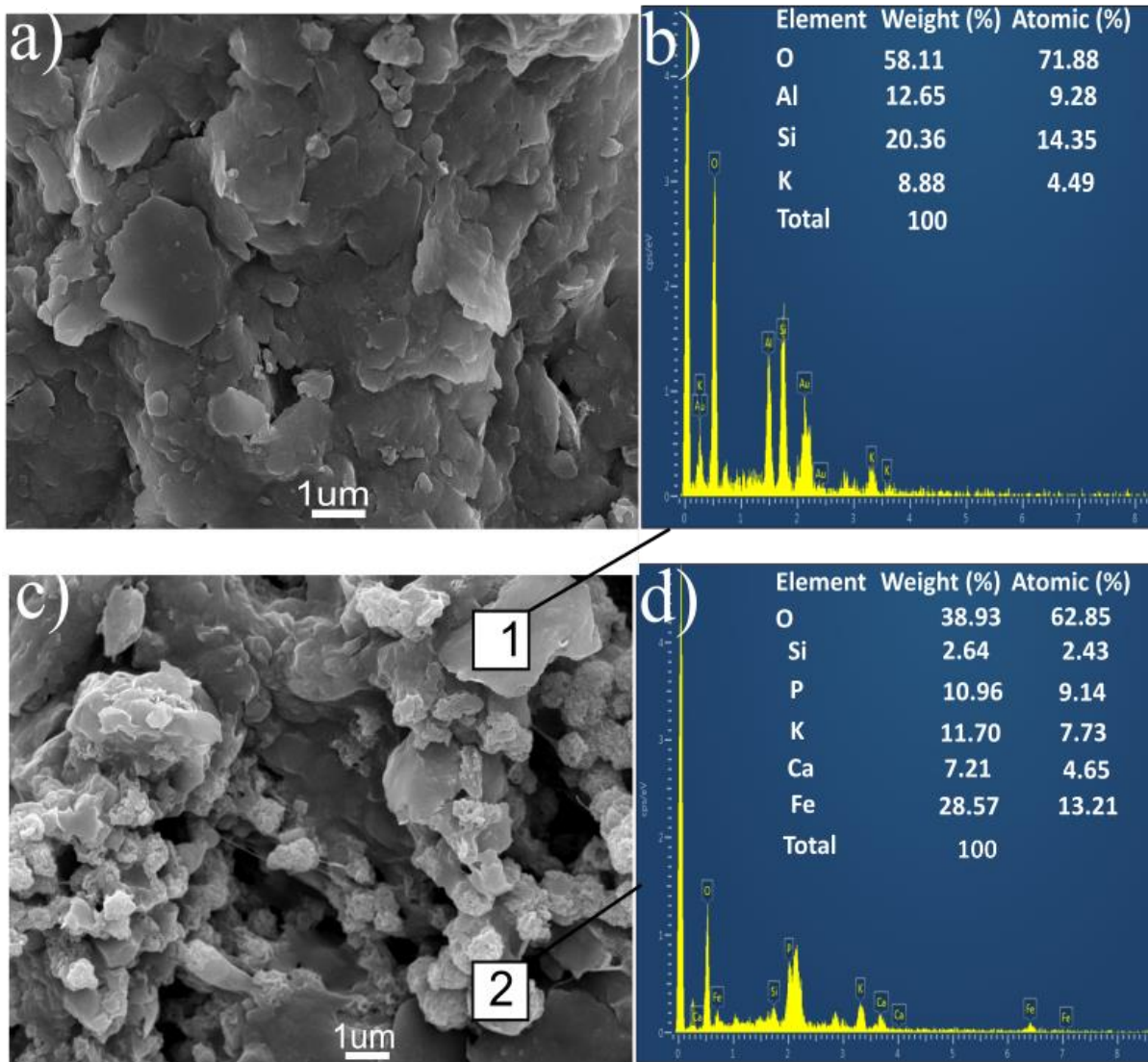
341



342 **Figure 4.** XRD analysis comparing the mineralogical assemblage in paleomarine sediment (PS)
 343 before and after treatment (PS/MFC).

344

345 Yet, the SEM images show the presence of new precipitation rich in iron and phosphorus (from
 346 phosphate buffer used as electrolyte) compared to the initial marlstone sample which is rich
 347 in aluminosilicate (Figure 5, Supporting Information Figure S16). The particle size ranges of the
 348 new precipitate phase and their low abundance would make this phase undetectable by XRD
 349 as previously reported [38].

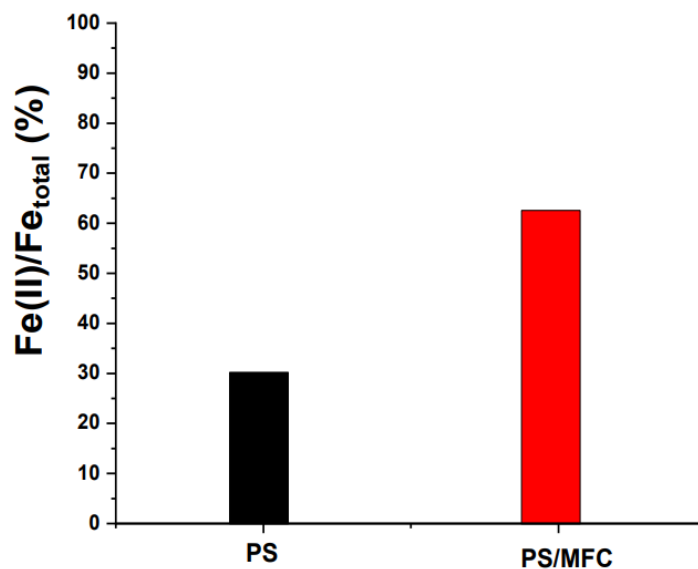


350

351 **Figure 5.** Scanning electron microscopy (SEM) images of the marlstones samples both before
 352 (a) and after (c) incubation (PS/MFC). a) displays the structure of clay minerals present in the
 353 PS before incubation. Small agglomerations with high concentrations of iron and potassium
 354 are observed in c) as confirmed from energy-dispersive X-ray spectroscopy (EDS) analysis (b)
 355 and d) that compares the initial composition of the PS (labeled as 1) with the composition of
 356 a new precipitate (labeled as 2). This analysis demonstrates a significant enrichment of iron in
 357 the newly formed precipitate.

358

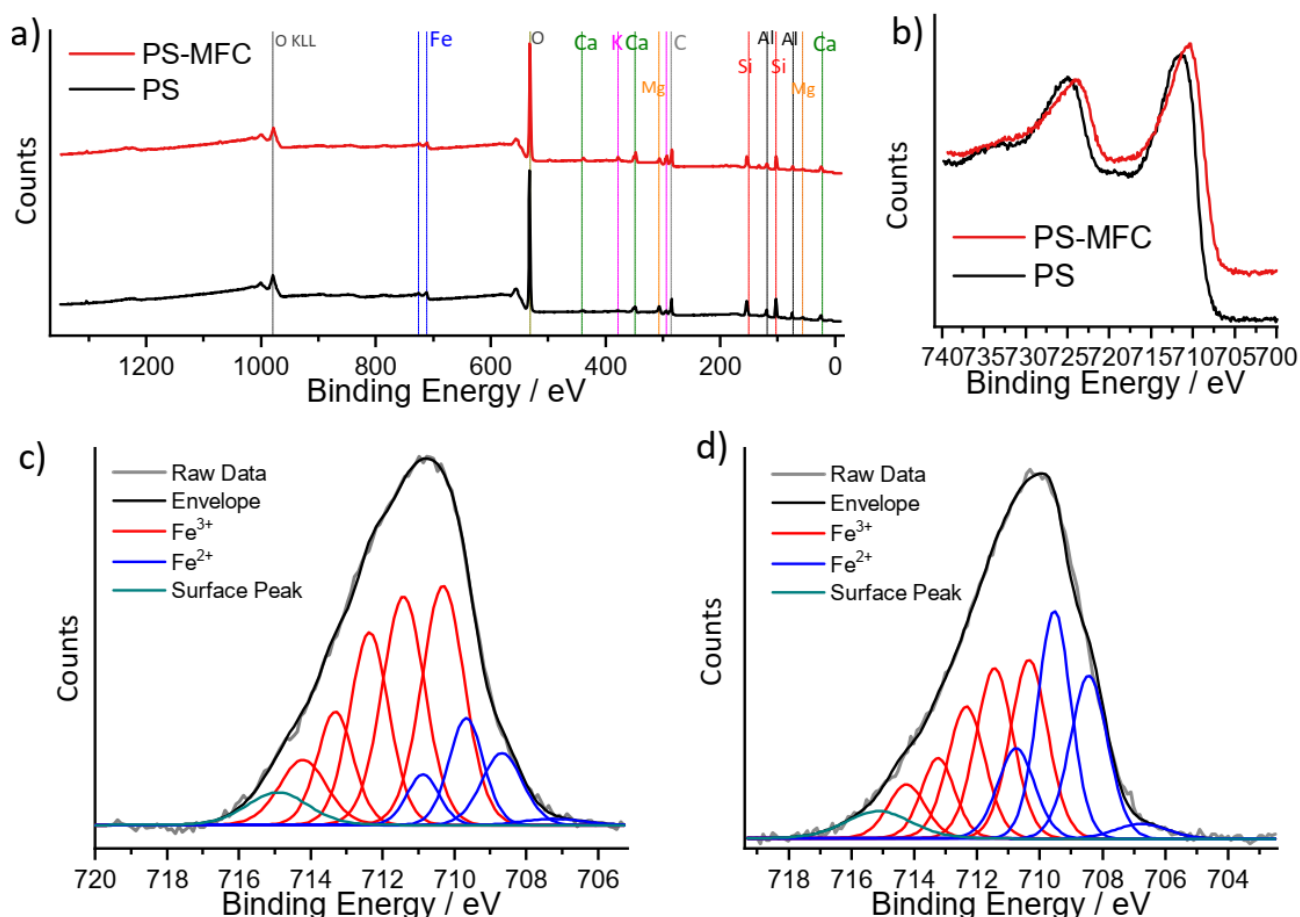
359 These results agree with our geochemical analysis of the powder before and after incubation,
360 where we recorded an increase on Fe_{total} and Fe (II). The potential link between the
361 development of exoelectrogenic bacteria in the PS/MFC system and the increase in the
362 concentration of ferrous iron Fe (II) is likely related to the reduction of Fe(III) present on the
363 clay minerals by the developed biofilm (Figure 6) followed by re-precipitation as oxide[39].
364 The interaction between bacteria and clay minerals is well-known in geochemical phenomena
365 [6]. The microorganisms have the ability to degrade and decompose clay minerals [40],
366 promoting the conversion of smectite to illite and the biotransformation of chlorite to
367 vermiculite [41]. Moreover, the bacteria have the ability to reduce structural iron Fe (III) of
368 clay minerals which play a main role in iron cycles in marine sediments [42] to Fe(II) as
369 documented for the species *Bacillus sp* isolated from clay minerals [43]. The distribution of Fe
370 in the layered structure can affect the chemical reactivity of clay since reduction or oxidation
371 of Fe will affect the layer charge and hence change the properties of minerals in terms of
372 cation exchange capacity and swelling [44] [37]. According to the literature and our data, we
373 hypothesize that the black powder developed in PS/MFC is related to the reduction of iron
374 Fe(III) present in the marlstone samples to Fe(II) by the developed exoelectrogenic bacteria.



375 **Figure 6.** Iron content of the marlstone samples powder before (in black) and after
376 incubation (in red)

377

378 After MFC operation there is an apparent increase in the contributions of potassium along
 379 with the appearance of a small phosphorus contribution in the survey, consistent with the use
 380 of potassium phosphate buffer in the electrolyte. No change in total iron content was found
 381 after MFC operation, however, changes in the oxidation state of the iron are evident in the
 382 high-resolution XPS spectra showing the Fe 2p_{3/2} and 2p_{1/2} peaks (Figure 7 b) which may be
 383 attributed to the reduction of Fe(III) to Fe(II) by electroactive bacteria.



384
 385 **Figure 7:** a) shows representative survey spectra of the PS and PS-MFC. Peaks associated to
 386 selected assigned elements are indicated above the spectra in each case. b) High resolution
 387 spectra in the region containing Fe 2p_{1/2} and 2p_{3/2} peaks after charge correction using
 388 adventitious carbon in the C 1s envelope at 284.8 eV. c) and d) show deconvoluted high
 389 resolution spectra in the Fe 2p_{3/2} region. Spectra are shown after Shirley background
 390 subtraction. Details of the assigned peak positions and full widths at half maximum are
 391 reported in the supporting information Table S2.

392 The predominant elements detected in the survey are carbon, oxygen, silicon, and aluminum
 393 with contributions from iron, calcium, magnesium, and potassium (Figure 7 a). The relative

394 proportions of each element quantified from the survey scans (Supporting Information Table
395 S1) after accounting for relative sensitivity factors are broadly consistent with both EDS (Figure
396 5) and chemical analysis results reported in Figure 6. After MFC operation there is an apparent
397 increase in the contributions of potassium along with the appearance of a small phosphorus
398 contribution in the survey, consistent with the use of potassium phosphate buffer in the
399 electrolyte. No change in total iron content was found after MFC operation, however changes
400 in the oxidation state of the iron are evident in the high resolution spectra showing the Fe
401 2p_{3/2} and 2p_{1/2} peaks (Figure 7 b) which may be attributed to the reduction of Fe(III) to Fe(II)
402 by electroactive bacteria.

403 Chemical analysis (Figure 6, Supporting Information Figure S17) confirmed the presence of
404 both Fe (III) and Fe (II) species which may exist as ionic species within clay minerals [45,46]
405 and also as oxide species, further complicating the deconvolution of high-resolution spectra
406 shown in Figure 7 c) and d). Hence, deconvolutions of the 2p_{3/2} peak were attempted based
407 on characteristic multiplets for Fe (III) and Fe (II) reported in the literature[47–50], using the
408 most prominent multiplet peaks associated to the Fe (III) and Fe (II) oxidation states. In
409 addition, following the fitting protocol of Grosvenor et al. in reference[47], a ‘pre-peak’ which
410 may also be associated to Fe(II) species and a ‘surface peak,’ which the authors attributed to
411 surface effects of reduced coordination of Fe in crushed powder preparations, were found to
412 be necessary to improve the fit. Good fits were obtained by applying this model to both PS
413 and PS-MFC with similar values of the full width at half maximum (FWHM) and spacing
414 between multiplets peaks, as reported in the Supporting Information Table S1 and S2. The
415 most notable difference in the PS-MFC after MFC operation is the significant increase in the
416 contributions of Fe(II) peaks in the region between 706-710 eV to the deconvolution.

417 Examining the relative peak areas for both samples yields estimates of Fe (II)/Fe(tot) of ca.
418 18% in PS and 40% in PS-MFC, in agreement with chemical analysis methods which suggested
419 that the Fe(II) proportion doubled in sediments isolated after 40 days of continuous MFC
420 operation. As XPS experiments and chemical analyses were carried out on the same samples,
421 the lower absolute value of the estimated Fe(II) content from XPS may be explained by the
422 overlap of multiplets associated to the two Fe oxidation states.

423 **Discussion and Conclusions**

424 Exoelectrogenic bacteria development has been previously demonstrated for a wide variety
425 of soil-based inocula, suggesting a broad predominance of these bacteria in anaerobic

426 environments where Fe(III) and other metal oxides found in common ores may serve as
427 alternative electron acceptors. Here we have shown that such bacteria may occupy a similar
428 niche in marlstone which possess comparably small levels of organic matter compared to soils
429 but are rich in clay minerals and iron. Bacteria of the genus *Geovibrio*, *Geobacter*, well known
430 for their electroactivity [20] [51] were the main electricigens identified and are proposed to
431 explain the observed catalytic currents as well as the bulk modifications observed in the
432 sediment inoculum in the MFC pilots. It is likely that the exo-electrogenic bacteria present in
433 these sediments play a role in the cycling of Fe(III) present not only as trace oxide particles but
434 also as ions encapsulated within the clay mineral interstices. This has implications for the
435 structural modification of marlstone by the known metabolic processes of electroactive
436 bacteria.

437 The sequencing data of the interface sediment solution shows the absence of exoelectrogenic
438 bacteria, suggesting that these bacteria are only capable of developing into extensive biofilms
439 under artificial selection conditions conducive to MFC development. Nonetheless, once
440 developed such biofilms were apparently capable of dramatically affecting the oxidation state
441 of all iron present in the clay mineral, a result which was not observed in control pilots not
442 connected to an external cathode. We speculate that observed bulk changes in sediment color
443 and composition identified via a combination of chemical and imaging analysis are directly
444 related to Fe(III) to Fe(II) reduction followed by subsequent reprecipitation of Fe(II) as small
445 aggregates identified by SEM-EDS. Further research is ongoing to evaluate whether direct or
446 indirect electron transfer processes are responsible for these bulk modifications as well as
447 tests of a range of clay mineral organic matter and iron concentrations. Nevertheless, the
448 results presented here strongly suggest that electroactive bacterial activity can be implicated
449 in the modulation of marine sediment structure and composition. It demonstrates that
450 microbial communities and clay mineral assemblages mutually interact by exerting a selection
451 pressure on the microbial community and reducing iron contained in clay minerals such as
452 smectite, illite and chlorite.

453

454 **Declaration of Competing Interest**

455 The authors declare that they have no known competing financial interests or personal
456 relationships that could have appeared to influence the work reported in this paper.

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467 **Appendix A. Supplementary data**

468 Supplementary Figures and Tables can be found in the attached file including a closer view of
469 Col de Pré-Guittard section, paleomarine sediment (marlstone) characterization, the bioanode
470 setup and the voltammograms of the bioanode before and after biofilm removing, additional
471 SEM images of the biofilms developed at the surface of the bioanode, supplementary data
472 related to the Average repartition of bacterial communities (phylum) extracted from the
473 interface solution-sediment and color alteration recorded at the PS/MFC after development
474 of the exoelectrogenic bacteria , supplementary Different SEM image of the PS/MFC showing
475 the presence of biogenic new precipitate rich on iron.

476

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