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Effects of pH variations on anodic marine consortia in a dual chamber microbial fuel cell

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Abstract

The effect of anodic pH on Microbial fuel cells (MFCs) inoculated with marine consortia was investigated to characterize the microbial community adaptation to possible pH environmental changes and to define the pH extreme boundaries beyond which MFCs do not run properly.

Tests were conducted in triplicate using different feeding pH values (pH_{feed}) ranging from 3 to 13. The MFCs inoculated with marine consortia had a strong self-regulation ability and actively counterbalanced small variations in pH_{feed} maintaining the pH inside the anodic chamber (pH_{anode}) close to neutrality.

As soon as the pH_{anode} deviated from neutrality it affected MFCs' performances. Alkaline conditions with pH_{anode} values between 8 and 10 corresponded to the formation of a denser biofilm together with the best performance in terms of maximum power density (Pmax). Conversely, when the pH_{anode} reached values lower than 5.5 or higher than 10, a sharp drop in MFC performances, as well as a decrease of viable population, were observed. Interestingly, the system was able to survive these extreme conditions and restart working effectively when neutrality was reset.

The obtained results underline the high adaptability and recovery ability of anodic marine consortia even in extreme conditions, suggesting the employment of this inoculum for MFC applications as biosensors for on-site seawater monitoring or as power supply units to be installed in remote area.

Keywords

Marine consortia; Anodic pH; Self-regulating ability; Anodic potential; Biofilm morphology

1. Introduction

Microbial fuel cells (MFCs) are bioelectrochemical systems that directly convert the chemical energy stored in organic compounds into electricity via metabolic processes of microorganisms [1]. MFCs are a versatile emerging technology, offering a broad range of biotechnological applications [2,3]. The greatest potential of MFCs lies in energy production from biomass or wastewater as fuel, combining wastewater treatment and energy recovery [4]. **Nonetheless, MFCs are gaining scientific and commercial interest for other types of applications such as bioremediation of contaminated areas and in-situ power generation for remote areas** [5]. Another promising application regards the use of MFCs as sensors for water quality monitoring: their operational simplicity and potential cost-effectiveness may be the answer to efficient water sensing devices in developing countries [6,7]. Similarly to other batteries or fuel cells, MFCs are constituted by two electrodes. Their peculiarity lies in the exoelectrogenic bacteria that reside at the anode (and sometime at the cathode[8,9]) forming a biofilm able to breakdown the biodegradable organic molecules present in the solution by performing biochemical oxidation reactions. The microbial biofilm is sensitive and reactive to external conditions, such as changes in environmental conditions and operation modes, which affect the current and power production of the devices. Especially for MFCs working on-site, temperature and pH are crucial for the microbial electroactive biofilm formation and for the charge transfer. Several research groups have investigated the effect of these parameters on MFC performances [10–13]. In particular, pH of the anodic chamber is one of the important factors that can influence both the optimal microorganisms growth and the substrate metabolic activity, **consequently effecting the electron and proton generation mechanisms** [14,15]. The literature provides different results based on the nature of the biofilm, on the initial pH during the startup phase as well as on the MFC configuration; among them, majority of the works indicate alkaline condition as the best one for improving MFC performances. However, many studies analyzed pH variation in batch [16–18] or fed-batch mode [19] and directly at a fixed pH [10,20], without considering how the biological system adapts to the slow and continuous variations caused by an unexpected polluted fluid stream that can temporarily modify the ecological equilibrium present in natural environments, e.g. seawater or river. Moreover, in these studies, the MFCs are usually stabilized with high concentration of phosphate buffer which represents a significant limitation for on-site applications [14]. The present paper proposes an analysis of MFC performances under different pH conditions in absence of phosphate buffer, and the evaluation of the adaptability of anodic marine consortia, in order to better define the pH variances which can be tolerated during MFC operation. **In particular we propose a new experimental approach, firstly developing and stabilizing the microbial community at neutral pH and then exposing it to a continuous and slow variation of pH, mimicking the way in which environmental parameters changes can occur in real applications.** In the present work synthetic seawater salt mix was used as sole anodic electrolyte to simulate a real seawater environment and to create suitable microelements condition for marine microorganisms growth.

2. Material and Methods

2.1 Anodic inoculum preparation

The inoculum was derived from seawater (Arma di Taggia, Imperia, Italy) and was taken at the interface between water and atmosphere. The seawater sample was inoculated into the anodic chambers of MFCs after an enrichment procedure, described in [21], in sterile conditions. Briefly, the fresh seawater sample was enriched in anaerobic flasks in three consecutive steps of 72 h each, at room temperature (24 ± 2 °C) and under gentle orbital shaking (150 rpm). The sterile medium contained 10 g/L sodium acetate, 10 g/L peptone and 3.3 g/L of commercial seawater salt mix (Reef, Kent Marine Salt mix). During enrichment, the bacterial growth was monitored measuring the optical density at 600 nm (OD_{600}) with a LAMBDA 35 UV/Vis Perkin Elmer spectrophotometer in order to identify the optimal growth-phase for the microbial sub-culture passage. Finally, the bacteria suspension was used as inoculum into the MFCs, with a ratio of 10% v/v of the total anode volume. All reagents were purchased from Sigma-Aldrich unless otherwise specified.

2.2 MFCs design and operation

Experiments were conducted in a two-chamber fuel cell (chamber volume 58 mL). A Cation Exchange Membrane (CEM, CMI-7000, Membranes International Inc., USA), was used to separate the two chambers. The anode and cathode electrodes consisted of a carbon felt of 38.5 cm² (Soft felt SIGRATHERM GFA5, SGL Carbon, Germany). Electrical contacts to the electrodes were made with graphite rods and an Ag/AgCl Reference Electrode was inserted into anodic compartment. In the Supporting Information (SI), a picture of the dual-chamber MFC used (Fig. S1) and a schematic design and image of the continuous mode MFC set-up (Fig. S2) are shown.

The experimental set-up consisted in 3 groups of MFCs: neutral, acidic and basic. Each group was made of 3 identical MFCs which were subjected to the same pH_{feed} . The test lasted 62 days and the different phases of the experiment are schematically shown in Fig. 1 and briefly described here: i) start-up phase (pH 7) to promote biofilm formation; ii) 1st phase (pH_{feed} 7, 11, 5) in order to investigate the adaptability of the system to pH variation; iii) Restart period (pH_{feed} 7) to reset neutrality; iv) 2nd phase (pH_{feed} 7, 13, 3) to identify the pH thresholds beyond which the system does not run properly; v) Final phase (pH_{feed} 7) to understand the ability of the system to recover from an intensive pH stress. After 56 days of operation, two MFCs of each triplicate were sacrificed for DNA extraction and Field Emission Scanning Electron Microscopy (FESEM) analysis as described in Section 2.3. One MFC for each pH condition continued to run until the end of the test (day 62).

When operated in continuous mode the MFCs had a hydraulic retention time (HRT) of 5 days (0.5 mL/h). The feeding solution was pumped using multiple channel syringe pumps (NE1600, New Era Instrument, USA).

98 The experimental tests were performed at ambient temperature, from 22 to 26 °C. The organic substrate and the
99 nitrogen source consisted of 1 g/L per day of sodium acetate and 1.25 g/L per day of peptone, respectively, dissolved
100 into diluted artificial sea water (3.3 g/L of Kent Marine Salt mix), in the absence of phosphate buffer. The ionic
101 conductivity was 14.1 mS/cm. A parallel abiotic test was performed under the same conditions and is described in S 3.

102 The pH modification of feeding solution (pH_{feed}) was obtained by gradually adding 2N NaOH and 2N HCl for basic and
103 acidic influents, respectively. The pH value inside the anodic chambers (pH_{anode}) was daily monitored by taking liquid
104 anodic samples (pH-Meter, BASIC 20⁺, Crison). The cathode compartment was filled with potassium ferricyanide (6.58
105 g/L) used as oxidant compound, dissolved into a phosphate buffer solution (8.2 g/L of Na_2HPO_4 and 5.2 g/L of NaH_2PO_4).
106 Fresh catholyte was continuously recirculated using a peristaltic pump (Peri-Star Pro 8 channel, USA) at a speed of 40
107 revolutions per minute (rpm), in order to guarantee a stable cathodic performance.

108 **2.3 Electrochemical and biological characterization**

109 The system was analyzed from the electrochemical and biological point of view. The MFC and anodic potentials were
110 acquired automatically either in open circuit or under external load (2.7 k Ω) conditions using a data acquisition unit
111 (Agilent, 34972A).
112

113 Linear Sweep Voltammetry (LSV) and Electrochemical Impedance Spectroscopy (EIS) measurements were performed
114 during each step of pH variation, in order to compare the cell performances at each pH range. All these experiments
115 were carried out with a multi-channel VSP potentiostat (BioLogic) in a two-electrode set-up configuration: a working
116 electrode was coupled to the anode and both counter and reference electrode were connected to the cathode.
117 Polarization curves were obtained by LSV using a scan rate of 1 mV/s. Current and power densities were normalized by
118 the surface area of the anodic electrode (38.5 cm²). EIS measurements were conducted at cell open circuit voltage
119 (OCV), with a small AC signal of 10 mV amplitude and 10⁻¹ – 10⁴ Hz frequency range. The experimental spectra were
120 fitted with an equivalent circuit [22] in order to quantitatively evaluate the internal resistances.
121

122 Chemical Oxygen Demand (COD) analysis was made by photometric determination (Photometer PF-12 Plus, Macherey-
123 Nagel GmbH &Co, Germany) of Chromium (III) concentration after oxidation with potassium dichromate/sulfuric
124 acid/silver sulfate and using Nanocolor kit (Test 0-28 and Test 0-29, Macherey-Nagel GmbH &Co, Germany). The samples
125 were prepared according to manufacturer's instructions.

126 Plate count tests were conducted to identify variances in population growth after pH variations. Samples derived from
127 different MFCs were diluted in sterile water to a final dilution of 10⁻⁶. 100 μL of the diluted samples were plated in
128 triplicate on microbiological medium (Tryptone 5 g/L; Yeast extract 2.5 g/L; Glucose 1 g/L; Bacteriological agar 12g/L
129 dissolved in synthetic sea water) and cultivated in aerobic conditions for 48 h at 30 °C.

130 Real time quantitative Polymerase Chain Reaction (rt-qPCR) analysis were performed on samples of both planktonic
131 liquid phase and anode biofilm for the following genera of microorganisms: Total Bacteria, Total Sulfate Reducing
132 Bacteria (SRB) and Total Sulfate Oxidizing Bacteria (SOB), *Clostridium*, *Geobacter*, *Shewanella* and Methanogens.

133 Genomic DNA extraction was performed with a commercial kit (UltraClean® Microbial DNA Isolation Kit, MO-BIO
134 Laboratories Inc., Carlsbad, CA) according to manufacturer's instructions. Before DNA extraction, biofilm samples were
135 subject to a pre-treatment: 1.25 g of wet anode electrode was washed twice with 4 mL of 0.9% NaCl. Supernatants were
136 centrifuged for 20 min at 10,000 rpm. Pellets were re-suspended in 0.9% of NaCl solution. Rt-qPCR was performed using
137 Opticon Monitor 3 Software and the rt-qPCR Chromo4 thermal-cycler (Bio-Rad, Hercules, CA). Gene targets, primers,
138 reagents and thermal protocol for bacteria and methanogens were previously described in [22,23].

139 FESEM (ZEISS Merlin) analyses were performed in order to characterize the biofilm attachment to the anodic electrode.
140 Prior to FESEM measurements, anode samples were fixed in 2% glutaraldehyde solution for 1 h, followed by ethanol
141 dehydration series (i.e., 30%, 50%, 70%, 80%, 90%, 100% EtOH, 15 min for each treatment, and then left in 100% EtOH
142 overnight). The samples were dried with hexamethyldisilazane (HMDS) with serial incremental solutions (20 min in
143 solution 1 part HMDS, 2 parts 100% Ethanol followed by 20 min in solution 2 parts HMDS, 1 part 100% Ethanol and
144 finally 90 min in solution 100% HMDS) and left few hours under chemical hood to let HMDS evaporate. The resultant
145 specimens were coated with platinum using a sputter coater (Q150TES from Quorum technology sputtering system)
146 operating at 50 mA for 38 s at room temperature and with a base pressure of about 8×10^{-4} mbar. The samples were
147 observed with FESEM at 5 kV.

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3. Results and Discussion

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3.1 Variation of pH inside the anodic chamber

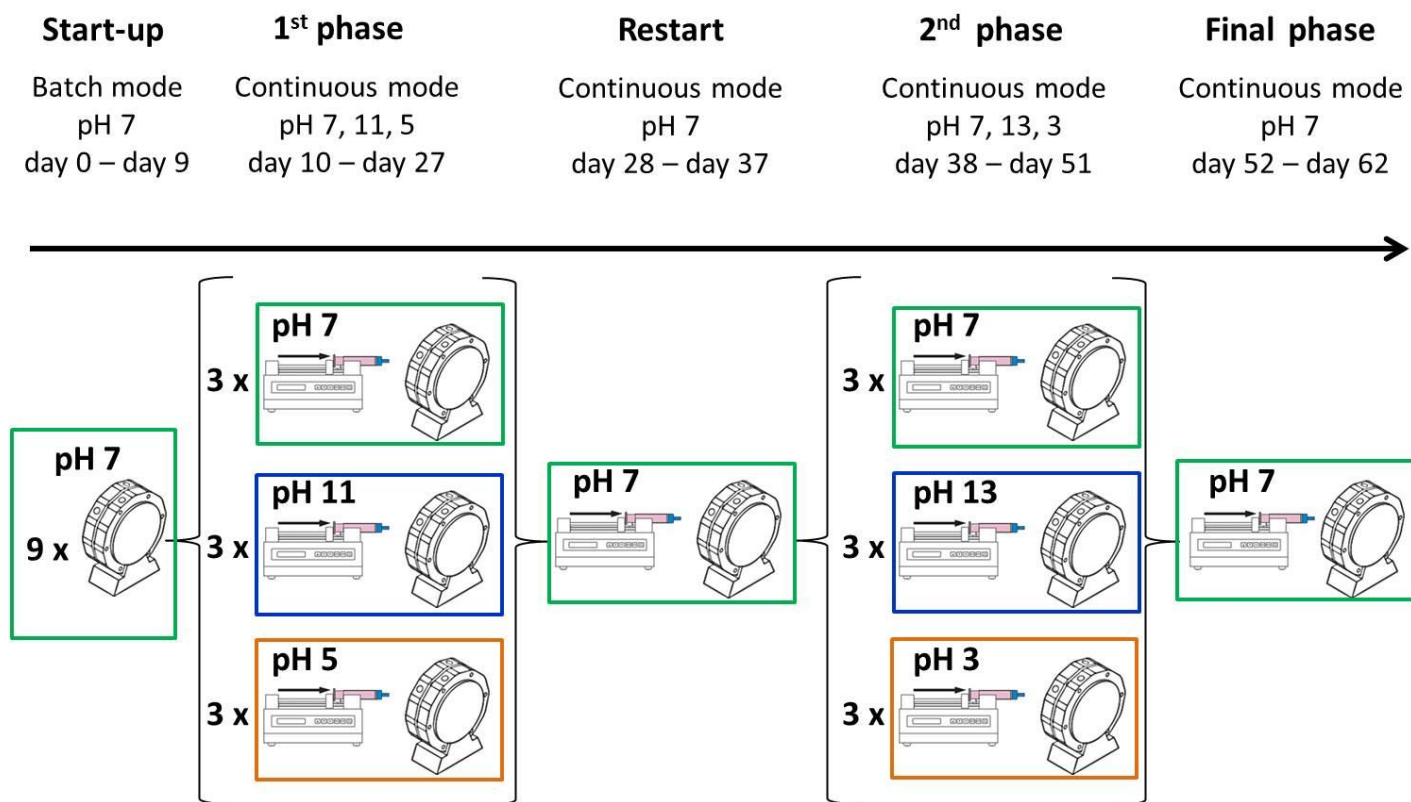
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In order to evaluate the microbial community adaptability to pH variances and to characterize the overall MFCs response, the devices were initially stabilized in neutral condition and consequently subjected to a continuous and slow variation of pH to mimic on-site application (Fig. 1).

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Fig. 1: Overview of the experimental phases.

161

i) Start-up phase: the 9 identical MFCs were kept in batch mode to promote initial biofilm formation;

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ii) 1st phase: MFCs were fed with influent at pH_{feed} of 7, 11 and 5 (3 MFCs for each influent) in order to investigate the adaptability of the system to pH variation;

164

iii) Restart: all the MFCs were subjected to the same influent at pH_{feed} of 7 to reset neutrality;

165 iv) 2nd phase: MFCs were fed with influent at pH_{feed} of 7, 13 and 3 (3 MFCs for each influent) with the aim to identify the
166 pH thresholds beyond which the system does not run properly;

167 v) Final phase: all the MFCs were subjected to the same influent at pH_{feed} of 7 to understand the ability of the system to
168 recover from an intensive pH stress.

169
170 In 1st phase of the test, the system demonstrated a strong self-regulation ability maintaining the pH_{anode} values close to
171 neutrality. In fact, as shown in Fig. 2, the pH_{anode} never reached the actual values of the influent (pH_{feed} of 5 and 11) even
172 after a complete replacement of the anolyte. Microorganisms may counteract changes in pH environment conditions
173 with diverse mechanisms of homeostasis including cytoplasmic buffering, adaptations of membrane structure, active
174 ions transport, and metabolic consumption of acids and bases [24]. In particular, marine heterotrophic bacteria have
175 maximum acidic and basic tolerance limits of 6.5 and 9.5, respectively [25], therefore other buffering mechanisms could
176 occur in the MFCs with a pH_{feed} of 5 and 11. For example, functional group of exopolymeric substances (EPS) secreted by
177 SRB exhibited different buffering capacities by releasing protons when the pH increases, and similarly, binding protons
178 when the pH decreases [27]. An identical experiment with abiotic MFCs was performed to exclude a buffering effect due
179 to the synthetic sea water, as shown in the SI (Fig. S4).

180 In 2nd phase, a drastic modification in pH of the feeding medium was performed (pH_{feed} of 7, 13 and 3) in order to
181 introduce an effective change of the resultant pH in the anodic compartment. Even though the values chosen for the
182 influent were extreme and poorly representative of naturally occurring circumstances, monitoring of the devices in such
183 conditions was helpful to understand the system itself and its equilibrium, and to investigate the effect of unpredictable
184 events such as unexpected pollutant streams. As a result of the alimentation with a pH_{feed} of 13 and 3, the pH_{anode}
185 reached the values of 12.5 and 4.2, respectively, revealing the inability of the bioelectrochemical system to counteract
186 pH variation when the influent is characterized by these extreme values (Fig. 2).

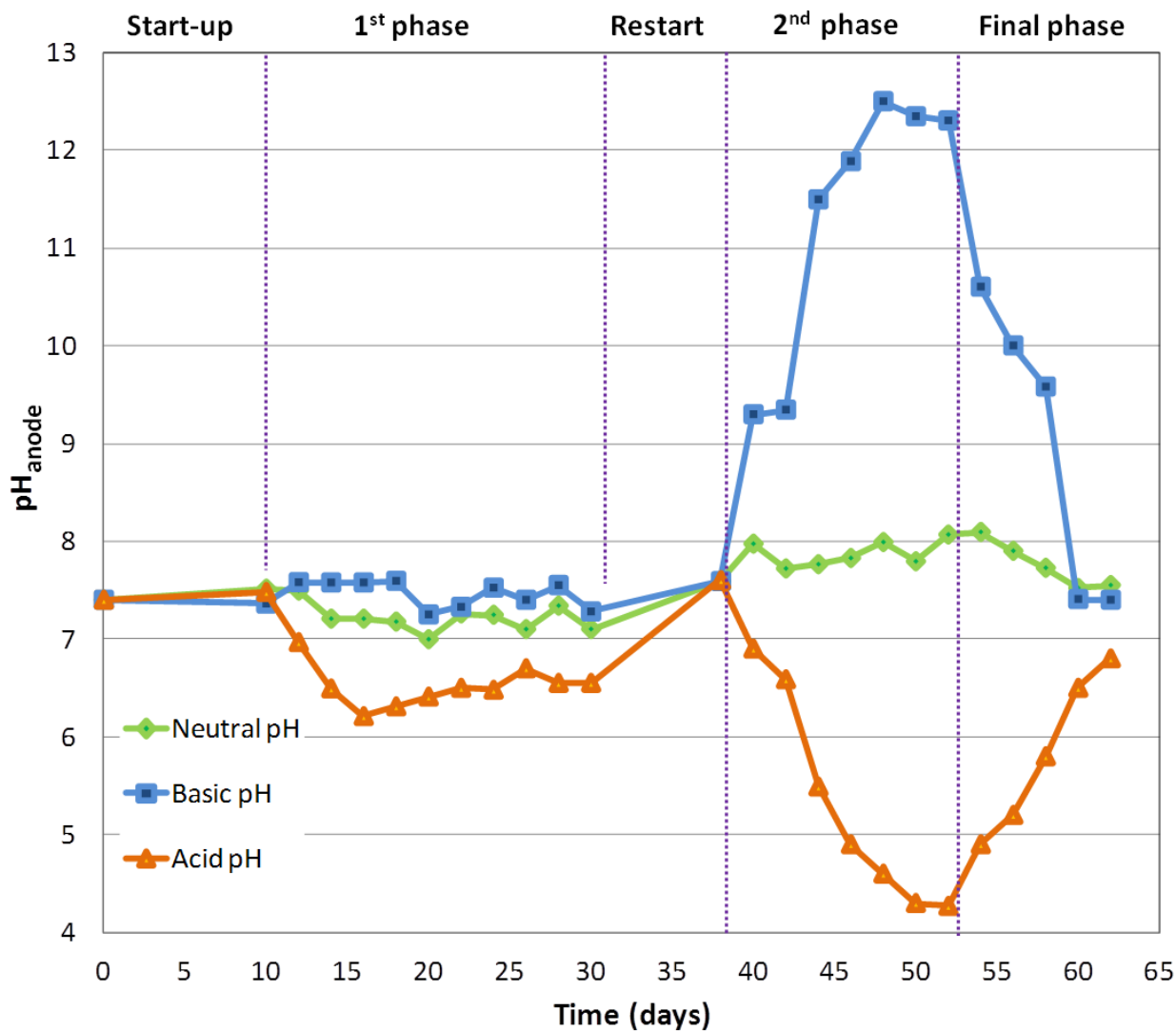


Fig. 2: pH_{anode} values measured into the anodic compartments. Data from 0-56 days represent the average of three MFCs subjected to the same pH_{feed} where the maximum variation observed within a triple for each pH point was 6.9%.

3.2 Effect of pH on MFC performances

MFCs performances were continuously monitored for each phase. As expected, in the first phase there were no substantial differences between neutral and basic MFCs (pH_{feed} of 7 and 11, respectively), since the resultant pH_{anode} was almost the same (Fig. 3a). In the case of acid MFCs (pH_{feed} of 5), a slightly negative effect of the pH on the performances was induced, demonstrating that even a small deviation from neutrality in the anodic compartment resulted in a reduction of the OCV (SI, Fig. S3) as well as P_{max} and short circuit current (I_{sc}) compared to neutral MFCs (Fig. 3a). In Figure 3 only a representative MFC among the triplicate is reported. However, in SI (Table S1) the average values of P_{max} and corresponding standard deviations are reported.

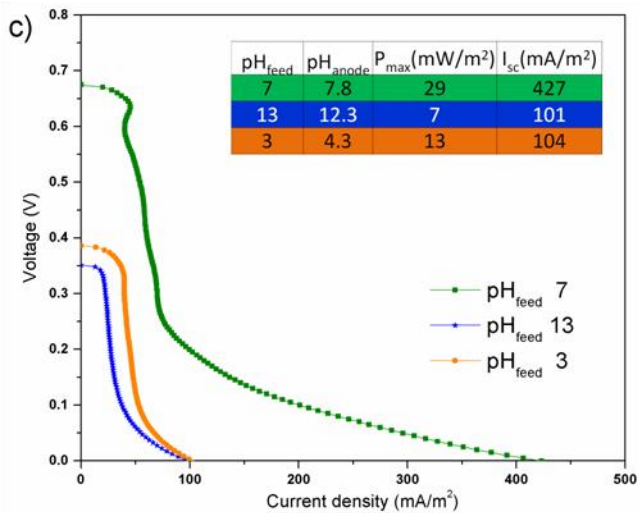
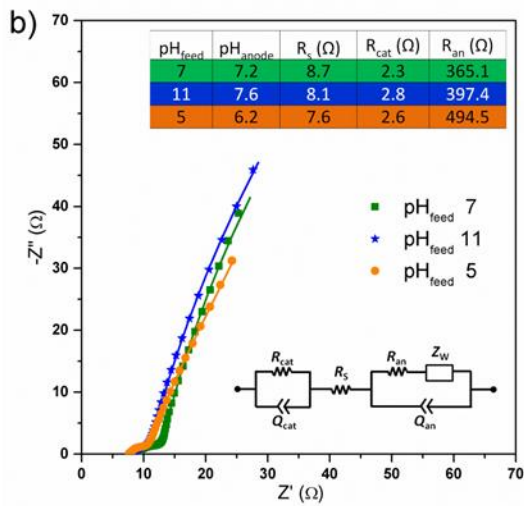
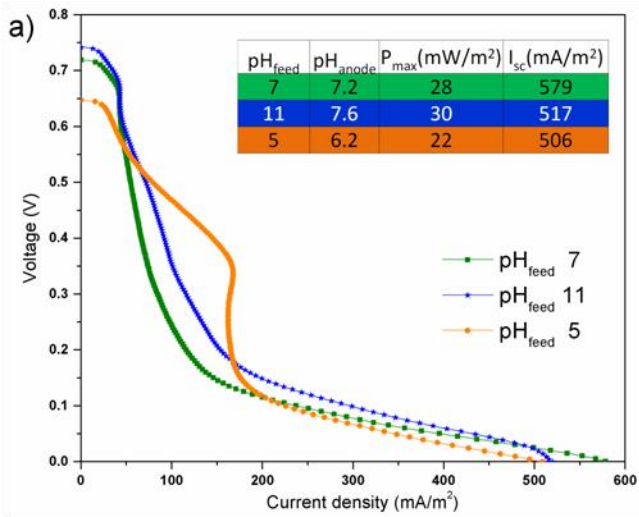


Fig. 3: Electrochemical trend of one representative MFC among the three subjected to the same pH_{feed} . a) Polarization curves at day 16 representing the cell voltage as a function of the current density. b) Impedance spectra at day 16: the points are experimental data, the continuous line are fitting curves (the inset shows the equivalent electrical circuit). c)

204 Polarization curves at day 48 representing the cell voltage as a function of the current density. The tables summarize
205 electrical parameters with respect to pH_{anode} actual values.

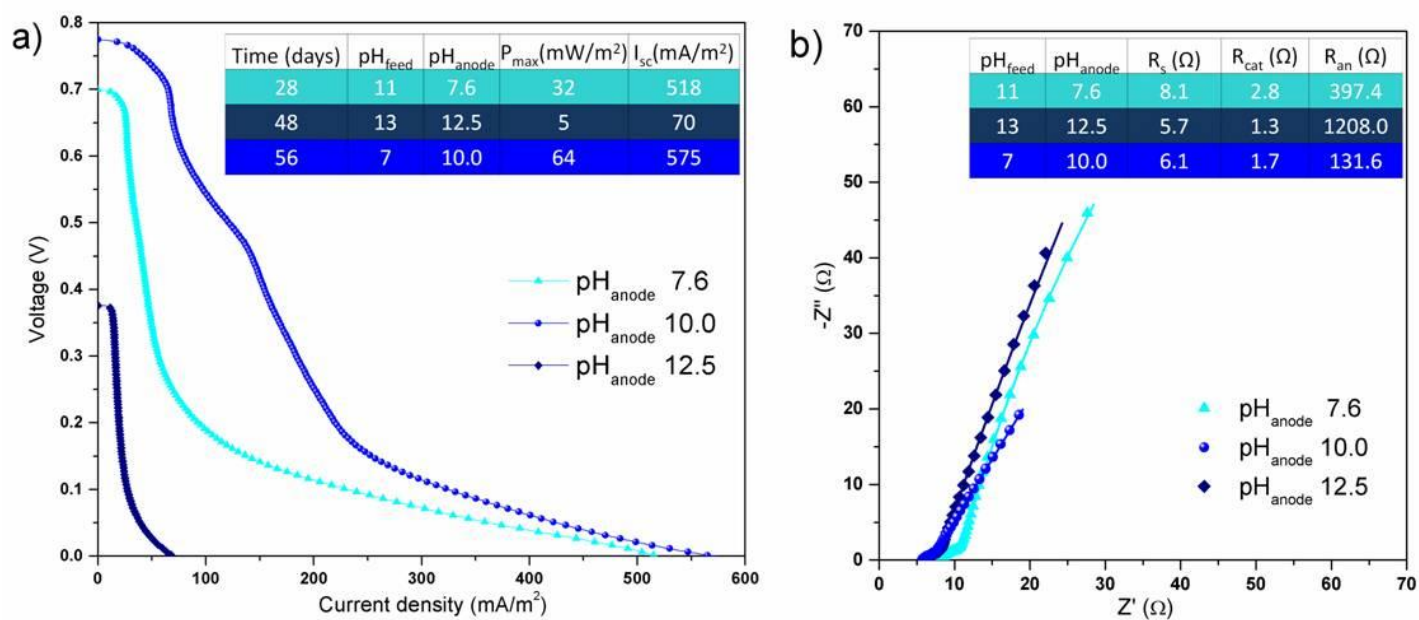
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207 The above reported results were successfully confirmed by the EIS analysis. The impedance spectra of three devices
208 subjected to different pH_{feed} , acquired at day 16, are reported in Fig. 3b. All the cells exhibited similar behavior, with a
209 shift on the real impedance axis proportional to the series resistance R_s (mainly due to the electrolyte and membrane
210 conductivities), a high frequency small arc proportional to the cathodic resistance R_{cat} (accounting for the charge transfer
211 at the cathodic electrode) and a larger (incomplete) arc proportional to the anodic resistance R_{an} (including the mass
212 transfer resistance) [22]. The spectra calculated through the fitting procedure (using the equivalent electrical circuit
213 defined in the inset) are also reported in Fig. 3b, superimposed to the experimental curves: the obtained electrical
214 parameters are summarized in the table of the same figure. Concerning the series resistance, it can be observed that
215 both acidic and basic devices exhibited slightly lower values if compared to the neutral ones, due to the presence of a
216 larger number of ions which increase the electrolyte conductivity [16]. On the contrary, as expected, similar values were
217 obtained for the cathodic resistances, since the cathodic compartment is less affected by the pH change of the anodic
218 influent. The main difference in the electrical parameters lies in the anodic resistances: in agreement with the result of
219 the polarization curves, the acid-based cells were characterized by a slightly larger resistance value (almost 500 Ω) if
220 compared to the other two devices (in the range 360 – 400 Ω), thus implying a reduced power production. This result is
221 consistent with the study of Behera & Ghangrekar [26], where a higher internal resistance for a device operating at pH 6,
222 with respect to a device operating at pH 8, was found.

223 In 2nd phase of the experiment, the inability of the system to counteract the pH variations was associated with a sharp
224 drop in the electrochemical MFC performances, both in basic and acidic MFCs. In particular, the pH_{anode} values lower
225 than 5 and larger than 10, represented the pH thresholds that nullify the self-regulation ability of the microbial
226 community (Fig. 3c). Extreme values reached in the anodic chamber negatively affected the performances of the devices
227 mainly because they determined an unsuitable condition for microorganisms survival and growth, as explained in the
228 next paragraph. Nevertheless, the system was able to survive extreme pH and restarted working normally at neutral
229 values, hence, showing a temporary and reversible condition which did not completely compromise the activity of the
230 device.

231 During the final phase, the pH was reset to neutrality and for basic MFCs it was possible to identify a window of optimal
232 pH_{anode} conditions. In fact, in basic MFCs pH_{anode} diminished from the maximum value of 12.3 down to 7.4, thus inducing a
233 strong increase in voltage and power production particular when pH_{anode} was in the range between 8 and 10. As shown
234 in Fig. 4a, the optimal pH_{anode} of 10 redoubled the maximum power density obtained with the same MFCs when pH_{anode}
235 was equal to 7.6. This behavior was in accordance with previous studies [27,28], but it has never been observed using
236 marine consortia as inoculum. The impedance spectra related to the cells fed with basic pH influent acquired at days 28,

237 48, and 56, as well as the fitted curves and the relative electrical parameters extracted after the fitting procedure
 238 (performed by using the same equivalent circuit already shown in the inset of Fig. 3b), are reported in Fig. 4b. By looking
 239 at these values, it can be observed a noticeable decrease of the electrolyte resistance while increasing the pH_{anode} , but
 240 also a reduction of the cathodic resistance, in agreement with the results reported by Liao et al. [28]. However, the
 241 major influence of the pH on the impedance was constituted by the anodic resistances, in fact, as the pH increased from
 242 7.6 to 10, the R_{an} values decreased. Similar results were obtained by Jadhav and coworkers [10]: they observed a
 243 decrease of the resistance while increasing the pH difference between the anode and the cathode. In the present work
 244 the cathodic pH remained quite constant due to the buffering effect of the catholyte and thus the increase of the pH_{anode}
 245 resulted in a larger pH difference between the anode and the cathode. Moreover, the effect observed on R_{an} can be
 246 attributed to an improved colonization of the anodic electrode (see the discussion below, Section 3.3), which was able to
 247 produce a larger current (and power), as reported in Fig 4a. By passing over the optimal basic pH range, an increase of
 248 the resistance was observed, likely related to the poorly viable biofilm (with a lower number of total bacteria, as
 249 discussed below), which was responsible for the reduction of the electric production, in agreement with the polarization
 250 curves reported in Fig. 4a.

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254 Fig. 4: Electrochemical trend of one representative MFC among the three subjected to basic pH_{feed} a) Polarization curves
 255 at different time points. b) Impedance spectra acquired at day 28, 48, and 56: the points are experimental data, the
 256 continuous line are fitting curves. The tables summarize the parameters with respect to pH_{anode} values.

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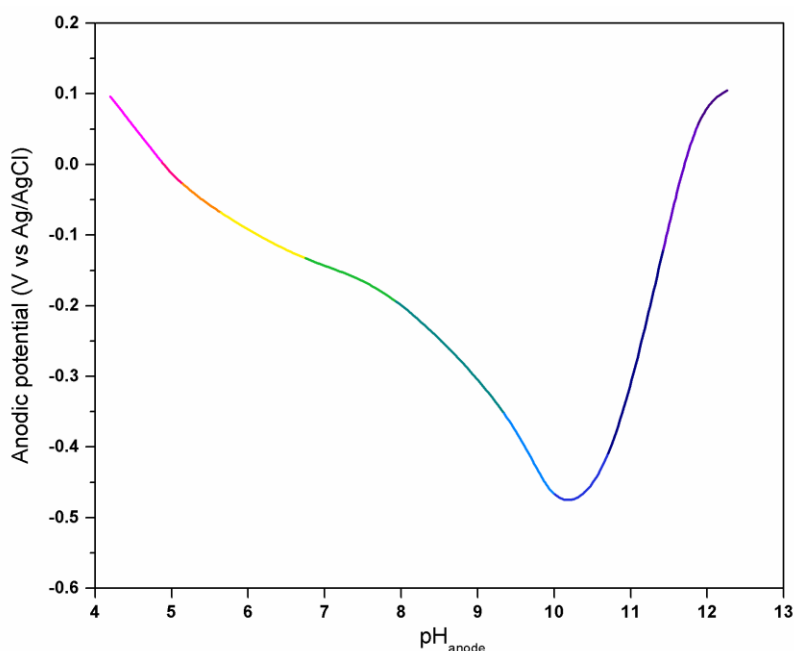
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To better characterize the devices performances under load, the individual electrode potentials were also measured. As shown in Fig. 5, the anodic potentials varied together with pH_{anode} conditions. For pH_{anode} values close to neutrality, the anodic potentials were around -150 mV, while the values were larger (i.e. more positive) for acidic pH_{anode} condition. The potentials observed are in accordance with Nernst equation which describes a shift equal to +59 mV for each pH unit moving from neutrality towards acidity [28,29]. Conversely, moving from neutrality towards alkalinity would induce a potential variation equal to -59 mV. In the present work, increasing the pH_{anode} until a value equal to 10 determined a decisive reduction of the potential, reaching values as low as -500 mV. Unexpectedly, for critical basic pH_{anode} conditions ($\text{pH} \gg 10$) the anodic potentials exhibited an increase, which brought it to positive values up to 100 mV when the pH_{anode} was equal to 12.3. This peculiar behavior cannot be explained solely with the Nernst equation. In fact, in the pH window recognized as the optimal one (pH_{anode} comprised between 8 and 10), the slope of the potential/pH curve doubled the predicted one: this result can be ascribed to the additional effect of the marine consortia, able to significantly increase exocellular electron pathways in these alkaline conditions. For pH_{anode} higher than 10, the expected potential reduction was not observed. On the contrary a strong increase was recorded probably due to the negative effect that such drastic pH can have on microbial metabolic activity and viability.



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Fig. 5: Anodic potential variation as a function of pH_{anode} .

To summarize, anodic chamber pH greatly affected the performances of the marine consortia-based MFCs. In particular, alkalization with an optimal pH_{anode} range between 8 and 10 increased the bioelectrocatalytic current production. On the other hand, pH_{anode} values lower than 5 or higher than 10 induced a sharp drop in MFCs performances. These results are in accordance with previous studies performed on other types of inoculum [19,25-27]. In addition, the present study

282 showed that the marine-based MFCs had the interesting ability to survive alkaline extreme pH conditions avoiding
283 irreversible microbial community degradation.

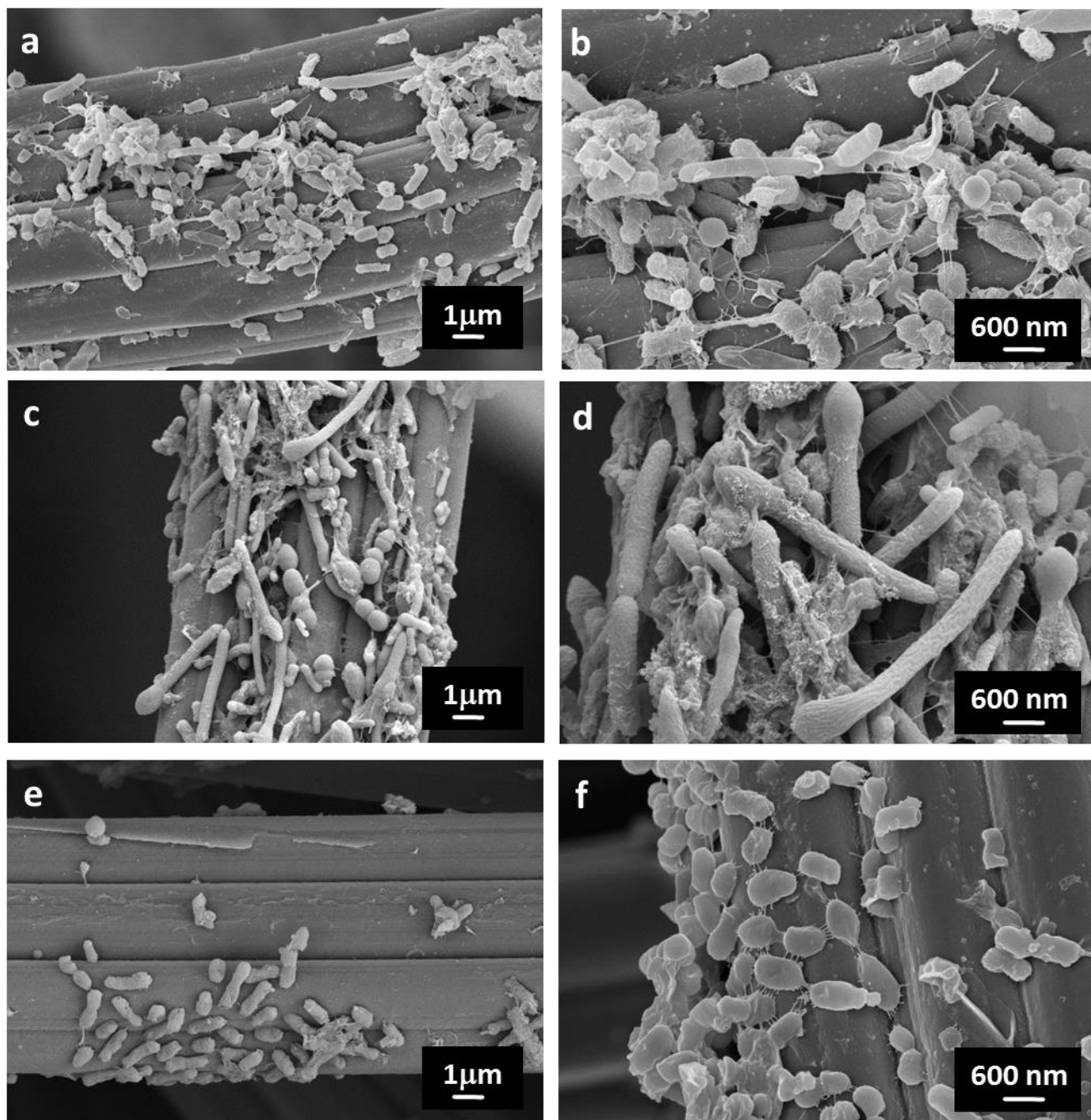
285 3.3 Biological characterization

286 To evaluate the correlation between microbial consortia viability, pH and MFC performances, planktonic samples were
287 analyzed during all the experiment, to determine the number of Colony Forming Unit (CFU) in agar plates. As
288 demonstrated by cells count, the different pH_{anode} had a direct impact on microorganisms growth which corresponded to
289 MFCs performances. In fact, a very low number of viable colonies were observed both in acidic and critical basic
290 conditions (pH_{anode} in the range 10.1-12.5), while the highest number of CFU was obtained from planktonic sample
291 derived from basic MFCs with optimal pH condition (pH_{anode} of 8-10), as shown in Fig. S5 in the SI.

292 The metabolic activity of the marine consortia as a function of the pH_{anode} was also evaluated by estimating the COD
293 removal efficiency. With pH_{anode} values close to neutrality (between 7 and 7.9) an average COD (η_{COD}) removal of 55%
294 was obtained. Lower COD consumption levels were observed for acidic MFCs (35%) and for pH higher than 10 (18%). In
295 spite of their better electrochemical performances, the basic MFCs with optimal pH_{anode} condition showed a η_{COD} equal
296 to 42% and therefore lower than what observed for neutral MFCs (see Fig. S6 in the SI). As already shown in [17] and
297 [30], especially while working with mixed population, COD consumption does not always follow the electrochemical
298 activity. In fact, a higher COD removal can be associated to substrate degradation performed by microorganisms for their
299 direct growth and sustenance, reducing the release of electrons contributing to current generation.

300 To better characterize the biofilm, its morphology was explored through FESEM at a single time point (day 56),
301 corresponding to pH_{anode} of 7.9, 10 and 5.2. Two out of three MFCs per different pH conditions were analyzed. As shown
302 in Figs. 6c and 6d, the basic MFCs presented a greater diversity in microorganisms morphology compared to neutral
303 MFCs (Figs. 6a and 6b). Moreover, the microbial community adhering onto the electrode was more densely connected,
304 probably thanks to the ubiquitous presence of EPS. The secreted matrix, which is well visible especially in Fig. 6d, not
305 only provides a scaffold for cell-cell interaction, but could also house electroactive components which enable bacteria to
306 transfer electrons to the anode [31].

307 On the other hand, as it can be observed in Figs. 6e and 6f, acidic MFCs showed a poorly attached biofilm with a lower
308 number of total bacteria.



309
 310 Fig. 6: FESEM images of MFC marine biofilm adhering onto anodic electrode in three different pH_{anode} conditions: neutral
 311 (a,b), basic (c,d) and acidic (e,f).

312
 313 Previous work suggested that electrochemical interactions between biofilm and anode are enhanced in alkaline
 314 conditions [16]. In present work the densely connected biofilm of the basic MFCs clearly contributed to the higher power
 315 output observed for these devices, as demonstrated by EIS results and also by the anodic potential monitoring over time
 316 (Fig. S3 in the SI). However, there are other elements that could have an additive effect in improving alkaline MFCs
 317 performances [32,33].

318 The **composition** of the marine consortia and its development at different pH was further investigated using rt-qPCR.
319 Both planktonic samples and electrodic materials (derived from 2 cells out of 3 at day 56) were subjected to DNA
320 extraction and then investigated for the presence of SRB and SOB, *Clostridium*, *Geobacter*, *Shewanella* and
321 Methanogens. In particular, these bacteria were chosen as markers of the phyla Proteobacteria and Firmicutes [34–36].
322 To analyze the microbial community and, in particular, the population dynamics, the initial inoculum was utilized as the
323 control [22]. The characterization performed using rt-qPCR identified the 49.3% of the total components, underlying the
324 high variability and diversity of the microbial marine community. With respect to the total bacteria, SRB and SOB were
325 the most abundant (48.8%) while *Shewanella* and *Clostridium* were present in small percentages (0.55% and 0.0001%,
326 respectively). In the present study Methanogens as well as *Geobacter* quantification by rt-qPCR resulted under the
327 resolution limit (< 250 gene copies/mL) both for inoculum and samples at day 56. The **limited** presence of bacteria
328 belonging to the Proteobacteria phylum (*Geobacter* and *Shewanella*) can explain the general low performances of the
329 presented devices independently from the pH variance. The comparison between the different MFCs at day 56 revealed
330 substantial variation in acid MFCs compared to neutral and basic ones. In particular, acid MFCs presented a lower
331 number of total bacteria and specifically a diminished number of SRB, SOB and *Shewanella*.

332 Neutral and basic MFCs had a very similar composition and the main difference was the higher number of total bacteria
333 in the biofilm derived from basic MFCs, which is in agreement with what observed through FESEM analysis. **These results**
334 **suggested that the analyzed genera are not sufficient to describe the complexity of the microbial activity inside the**
335 **devices and probably other aerobic genera, not usually presents in MFCs, can directly or indirectly affect the**
336 **bioelectrochemical behavior in these marine consortia-based MFCs.**

337 **4. Conclusion**

338 The aim of the current study was to investigate the response of marine consortia-based MFC at different pH determining
339 the pH extreme boundaries for irreversible biologic degradation, and characterizing the devices adaptation to pH
340 changes.

341 The results revealed that pH became a disturbing operating parameter only when pH_{feed} reaches extreme values. In fact,
342 pH_{feed} of 5 and 11 did not significantly modify MFCs performances, as demonstrated in the first part of the experiment.
343 Influent with critical pH values (i.e. pH_{feed} of 3 and 13) were used to effectively change the pH inside the anodic
344 chamber and to analyze the corresponding variation of MFCs performances. The optimal conditions for MFCs operations
345 in terms of current and power production corresponded to a pH_{anode} in the range between 8 and 10. On the contrary,
346 acidic conditions, as well as alkaline ones with a pH_{anode} higher than 10, induced a sharp drop in the electrochemical
347 performances. **Interestingly, the data highlighted that the critical pH reached inside the anodic chamber (namely, highest**
348 **12.5 and lowest 4.2 pH_{anode} values) did not irreversibly compromise the devices. In fact, the microbial community was**
349 **able to survive these unfavorable conditions and to re-develop an active biofilm when the pH_{anode} moved towards**
350 **neutrality.** The analysis of the different types of characterization performed with respect to pH variations suggested that

351 the densely connected biofilm observed in the basic MFCs with optimal pH_{anode} range was a key player for the higher
352 power output.

353 The possibility to work in a wide pH range, together with the ability to survive extreme pHs without any external
354 maintenance, such as the introduction of new inoculum, suggest the applicability of these type of devices as bio-
355 detectors for remote area sensing.

357 **Acknowledgement**

358 The authors are grateful to the members of the BioEnergy group for the constructive discussions. [In particular we are](#)
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511 **Effect of pH variations on anodic marine biofilm in a dual chamber microbial fuel cell**

512 **Supplementary information**

513 Valentina Margaria ^{*,#,1}, Tonia Tommasi ^{*,#,1}, Simona Pentassuglia¹, Valeria Agostino¹, Adriano Sacco¹, Caterina
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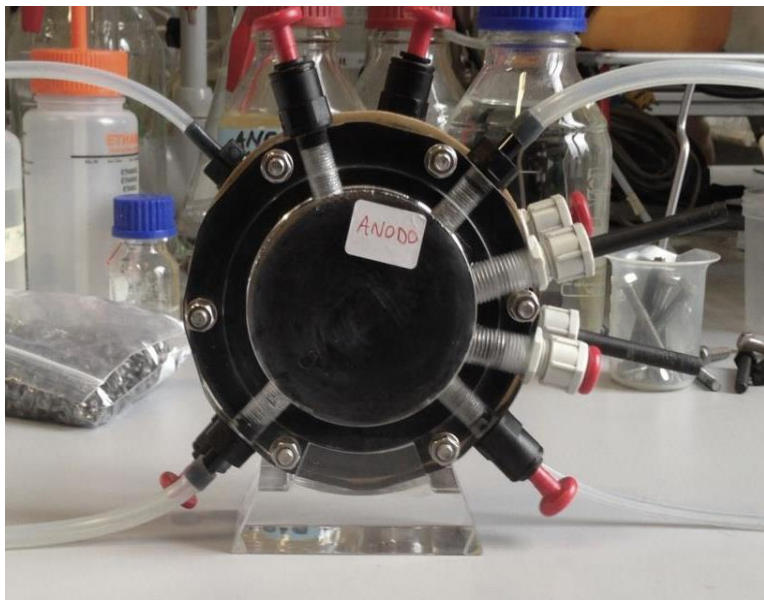
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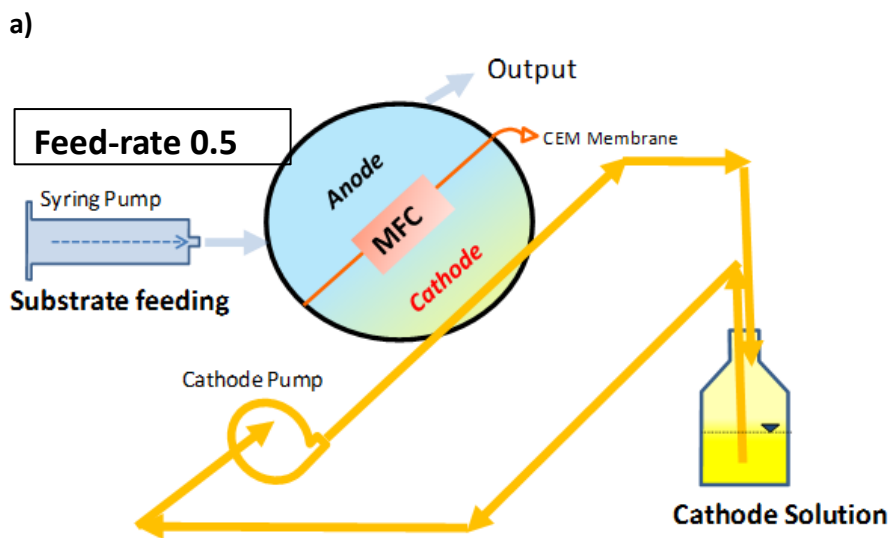
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520 **MFC design**

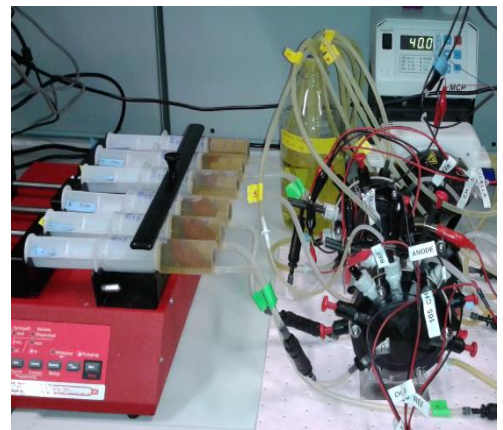


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531 **Fig. S1: Picture of the dual-chamber Microbial Fuel Cell (MFC).**

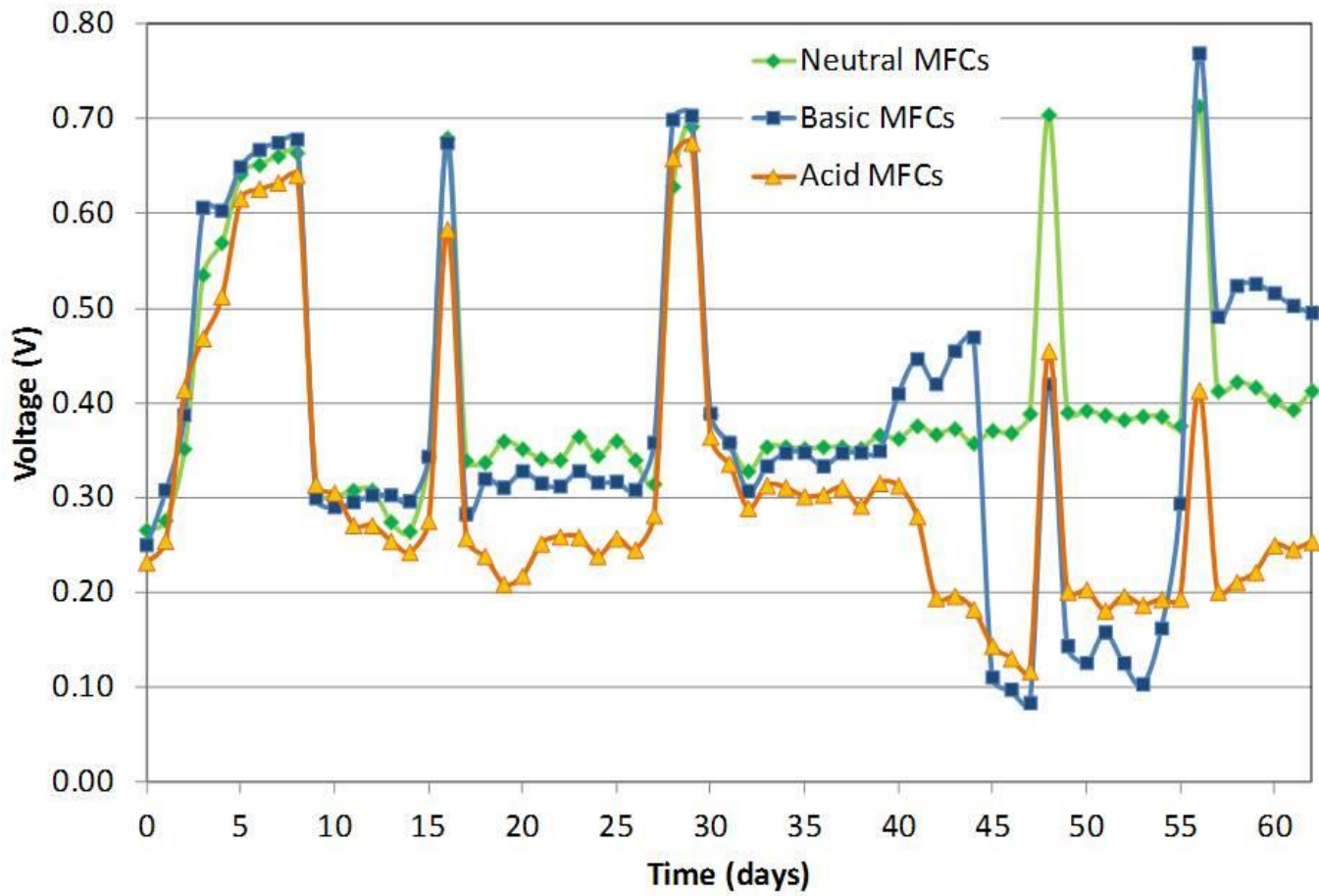


b)



541 **Fig. S2: a) Schematic diagram and b) picture of continuous mode MFC set-up. On the left side syringe pump for anolyte**
542 **feeding; on the right side peristaltic pump for catholyte recirculation. (Figure S2 was rearranged from [22]).**

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546 Fig. S3: Voltage monitoring over time. Data represent the average of three MFCs subjected to the same pH_{feed} . One
547 point per day (at the same hour) was considered and the maximum variation observed within a triple for each voltage
548 point was 16.2%. The peak at higher voltages corresponds to OCV conditions, during the start-up phase and before the
549 polarization analysis.

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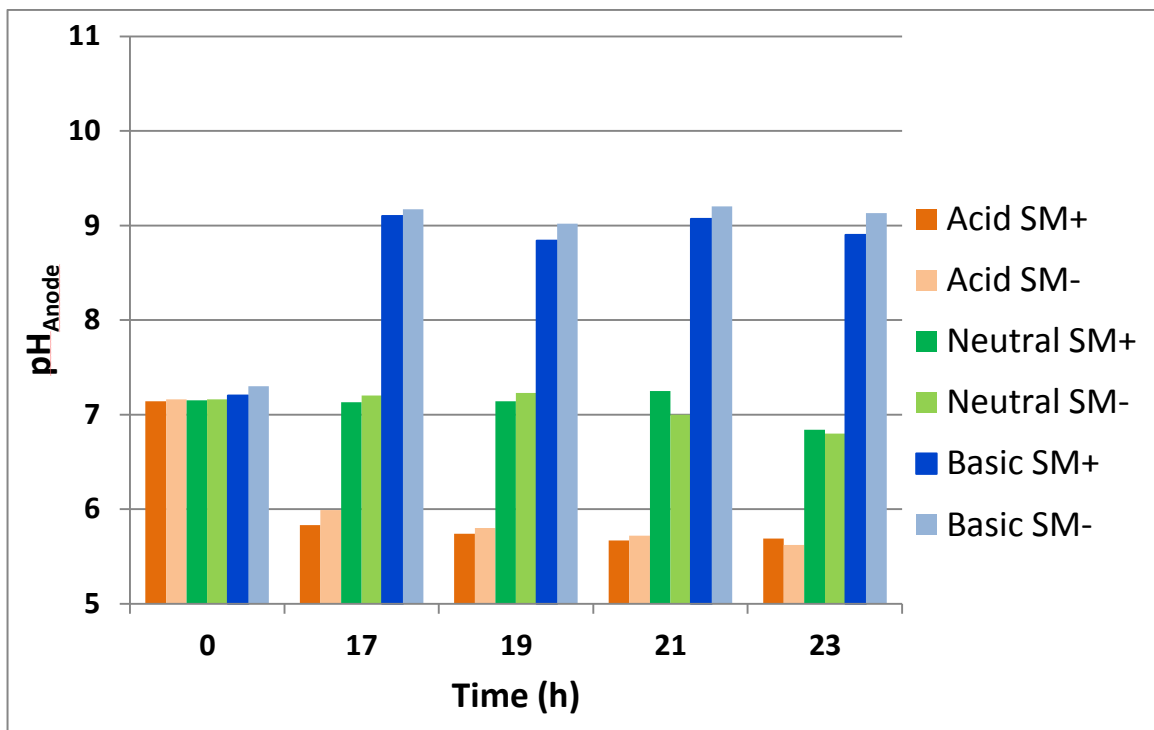
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559 **Abiotic test**

560 During the first phase, a parallel abiotic test was performed under the same pH conditions to evaluate and rule out
561 feasible synthetic sea water buffering effect. In detail, six abiotic MFCs were employed for this experiment. Three of
562 them were fed with anolyte medium described in material and method, and were denoted as SM+. Three of the six
563 MFCs were instead exposed to the same medium composition without the Salt Mix solution (SM-).

564 As shown in Fig. S4, significant anodic pH variations were observed in the abiotic anode chamber, in spite of no total
565 replacement of anolyte solution. After few hours (17 h) of continuous feeding with pH_{feed} of 5 and pH_{feed} of 11, pH_{Anode}
566 reached values of about 5.8 and 9.2, respectively both in presence and absence of artificial sea water. [These data](#)
567 [demonstrated that almost near neutral pH values measured in the biotic test were directly determined by the self-](#)
568 [regulation ability of marine microorganisms.](#)

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Fig. S4: Monitoring of pH_{Anode} variations as a function of time in abiotic MFCs, in presence (SM+) or absence of (SM-) of Salt Mix solution.

	Time (days)	pH _{feed}	pH _{anode} average	pH stdev	P _{max} (mW/m ²) average	P _{max} stdev
Neutral MFCs triplicate	16	7	7.2	0.05	27.7	2.8
	28	7	7.3	0.42	27	4.7
	48	7	7.9	0.23	24.2	4.2
	56	7	7.8	0.19	27.5	4.3
Basic MFCs triplicate	16	11	7.6	0.19	32.7	4.2
	28	11	7.5	0.09	33.3	8.1
	48	13	12.5	0.04	6.6	1.4
	56	7	10	0.13	57.5	5.4
Acidic MFCs triplicate	16	5	6.5	0.06	21.6	6.1
	28	5	6.4	0.23	20.2	3.9
	48	3	4.6	0.36	14.5	3.5
	56	7	5.2	0.21	16.1	3.2

Table S1: Average and standard deviation values of pH_{anode} and P_{max} obtained during polarization analysis.

Colony Forming Unit

Figure S5 shows the average number of CFU derived from planktonic samples collected during the different operational conditions. In particular the neutral MFCs (pH 7/7.9) represent the CFU control number to be compared with the results obtained from Basic MFCs with optimal pH condition (pH 8/10), Basic MFCs with critical pH condition (pH 10.1/12.5) and Acid MFCs (pH 6.9/4.2).

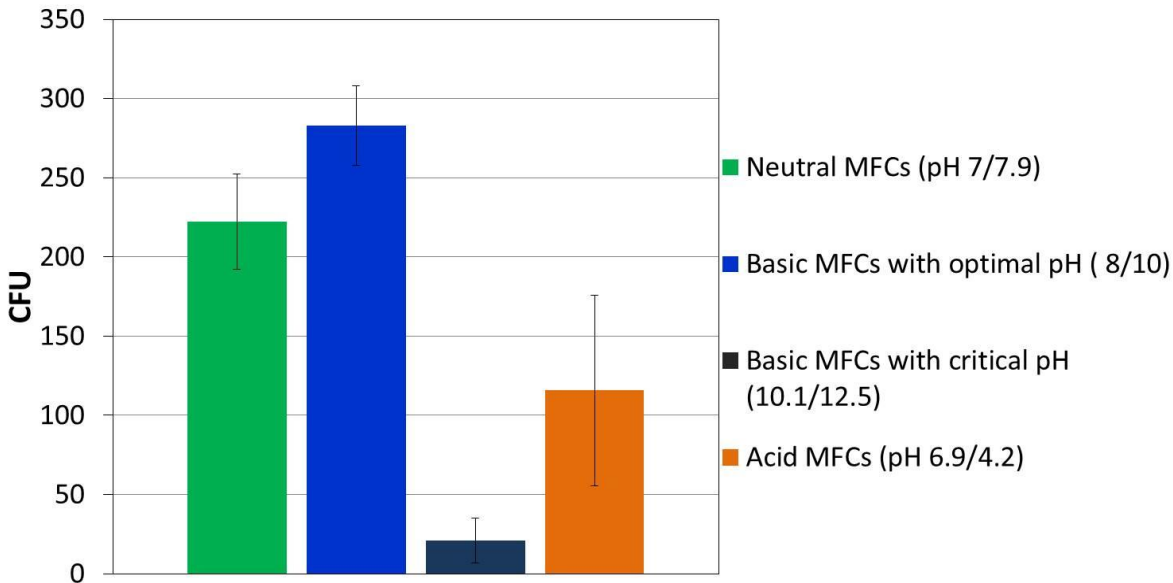
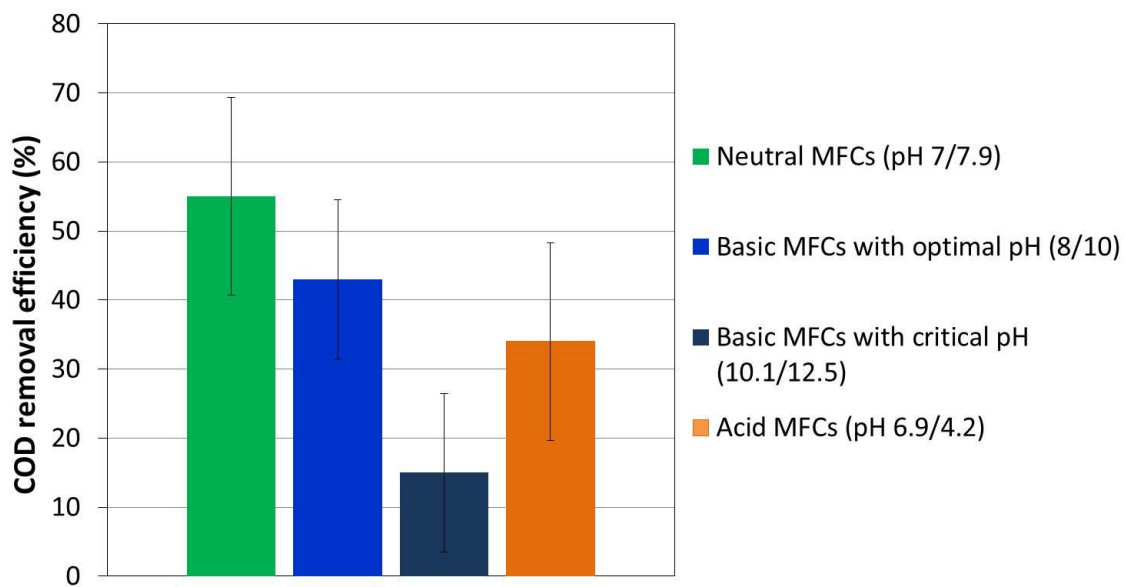


Fig. S5: Colony Forming Unit (CFU) on agar plates grown in aerobic conditions. Average of 3 MFCs subjected to the same pH_{feed}.

590 Chemical Oxygen Demand

591 Organic matter removal as a function of pH was evaluated by estimating Chemical Oxygen Demand (COD) removal
592 efficiency. The COD of the solution fed by syringe pump was 11.7 ± 0.3 g/L for all the pH_{feed} . In figure S3 a bar graph with
593 the different values of COD consumption is reported.



594
595 Fig. S6: COD removal efficiency (%). Average of 3 MFCs subjected to the same pH_{feed} .
596

Effects of pH variations on anodic marine consortia in a dual chamber microbial fuel cell

Valentina Margaria ^{*,#,1}, Tonia Tommasi ^{*,#,1}, Simona Pentassuglia¹, Valeria Agostino¹, Adriano Sacco¹, Caterina Armato^{1,2}, Angelica Chiodoni¹, Tiziana Schilirò², Marzia Quaglio¹

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Highlights

- Continuous and slow variation of pH_{anode} to mimic environmental changes
- Modest pH modifications were actively counteracted by the system self-regulating ability
- The optimal pH range for marine based MFCs operation was between 8 and 10
- Marine consortia was able to survive after drastic pH modifications

Turin, 18th July 2016

To the Editor:
Prof. Angelo Basile
International Journal of Hydrogen Energy
ELSEVIER.

Ref.: **Replies to Reviewer's Comments and Revised Manuscript to International Journal of Hydrogen Energy HE 2016 1187**

Dear Editor,

We would like to thank you and the Reviewers for the thorough evaluation that give us the possibilities to improve the quality of our work. Given below are the answers to the specific questions raised by the Reviewers and the comments to their suggestions.

For the benefit of the Reviewers, all the changes made in the revised version of the paper are highlighted in blue in the manuscript.

We hope that the paper could now be suitable for publication.

Sincerely,

The corresponding authors, on behalf of all co-authors.

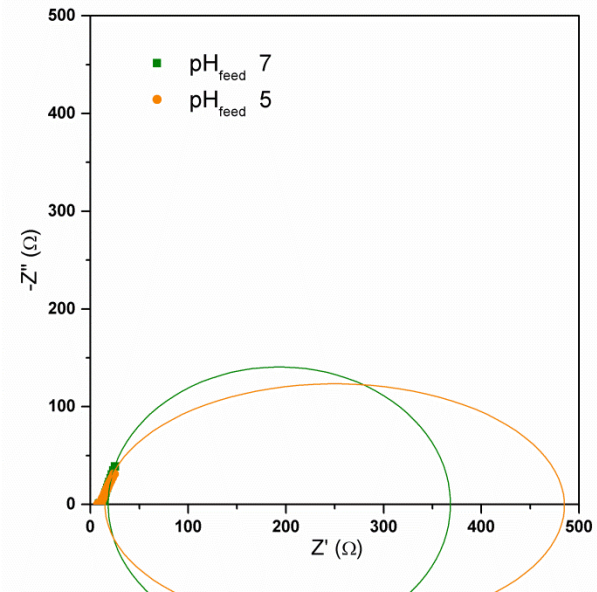
Valentina Margaria
Tonia Tommasi

Ms. Ref. No.: HE_2016_1187

Reviewers' comments

<p>Reviewer 1</p> <p>The manuscript entitle of " Effect of pH variations on anodic marine biofilm in a dual chamber microbial fuel cell " (Manuscript Number: HE_2016_1187)., by Valentina Margaria et al, depicts the effect of anodic pH on MFCs inoculated with marine consortia was investigated in order to define the pH extreme boundaries beyond which MFCs do not run properly, and to characterize the biofilm adaptation to possible pH environmental changes. Before the manuscript deems acceptable for publication, it needs be revised very well. Some of specific suggestions are listed below:</p>	
<p>1- The authors must be rechecking the English of the manuscript. It is fairly poor and must me improvement before publication by native person.</p>	<p>Accordingly, the manuscript was rechecked by a native English speaker</p>
<p>2- The authors must be more highlight in abstract section.</p>	<p>The abstract section was revised</p>
<p>3- What means the final sentences of abstract section? " biosensor for on-site seawater monitoring or power supply for remote area"</p>	<p>The final sentence in the abstract section was reformulated to clarify the meaning.</p>
<p>4- Why the inoculum was taken at the interface between water and atmosphere?</p>	<p>The choice of inoculum taken at the interface between seawater and atmosphere was made in order to see how a resident population tolerant to oxigen (all bacteria, and not specific ones) can electrochemically behave inside an MFC. In particular, we are involved in projects where MFCs power either electronics or sensors integrated into autonomous underwater vehicle that can operate at the sea surface as well as at different depth. For this reason, it was interesting to investigate the behavior of the selected inoculum into the MFCs. Moreover, this was not the first time that we used an inoculum sampled in the same way: in Tommasi et al., 2016 the presence and the enrichment of electrogenic microorganisms, such as <i>Shewanella</i> and <i>Geobacter</i>, was demonstrated.</p> <p style="text-align: right;">Tommasi T. Sacco A., Armato C., Hidalgo D., Millone L, Sanginario A., Tresso E., Schilirò T., Pirri C.F., Dynamical analysis of</p>

	<p>microbial fuel cells based on planar and 3D packed Anodes, Chemical Engineering Journal (2016) 288: 38–49.</p>
<p>5- The authors used mixed culture in their study. Why they selected sterile conditions?</p>	<p>The tests were carried out under sterile condition to avoid cross-contamination from different environmental samples used in our laboratory. Moreover, operating in this sterile condition, let us to attribute electrochemical performances to the starting mixed culture.</p>
<p>6- The authors talked about bacterial growth in material and method section but they did not present or talk about it in their manuscript at all.</p>	<p>As described in section 2.1, we estimated bacterial growth measuring the Optical Density at 600 nm (OD_{600}) during the inoculum enrichment steps. OD_{600} was determined to identify the optimal growth-phase for the microbial sub-culture passage. No measures were performed on samples taken from working MFCs. We improved the description in section 2.1 to avoid any confusion.</p>
<p>7- What was the HRT for cathode chamber for ferricyanide.</p>	<p>We chose a catholyte recirculating system using a peristaltic pump to ensure a stable cathodic performance. The catholyte was placed in a 500 ml glass bottle as reservoir and recirculated through the cathodic chamber at a speed of 40 rpm. This operational mode included the replacement of the recirculating catholyte solution every 72 h.</p>
<p>8- From the figure 3 b, it can be understand the pH 5 has in contrary with data provide inset.</p>	<p>As it is reported in the figure here below, the resistance values are strictly related to the slope of the Nyquist arcs, in the sense that an arc whose initial slope is steeper (green curve in the figure below) will lead to a resistance which would be lower with respect to an arc with lower steepness (orange curve), no matter of the $-Z''$ values at a certain frequency (in Fig. 3b the last points of all the three curves were acquired at 100 mHz), which is instead related to the capacitance behavior. For this reason, being the pH5 curve characterized by lower steepness with respect to the other two curves shown in Fig. 3b in the manuscript, its resistance should be larger, and this is in accordance</p>

	<p>with the values obtained by the fitting procedure, as reported in the table (Inset in Fig. 3b).</p> 
<p>9- Figure 1 is not informative and must be improve</p>	<p>We revised the Figure 1 adding a brief description of each phase to clarify the experimental process.</p>
<p>10- The authors must be provide EIS circuit mode in the figure of EIS spectra as an inset.</p>	<p>The equivalent electrical circuit used for the fitting of the impedance spectra was added as an inset in Fig. 3b. The figure caption was modified accordingly.</p>
<p>11- Schematic and photograph image of MFC is necessary in supplementary material section.</p>	<p>A schematic of the MFC and recirculating system as well as photographs were added in the supporting information.</p>
<p>12- One of important parameter on MFC performances is columbic efficiency.</p>	<p>According to the Reviewer's comment, we have calculated Coulombic efficiency (CE), that results in all case very low (<1%). We associated this low value mainly to the high external load used during the test that brings to low current recovery, considering that we were working near Open Circuit Condition. Even if low, the trend reflects the behavior of acid, basic and neutral MFCs, that is that acid MFCs have worse performances than neutral and basic MFCs. However, the aim of our work was mainly focused on the effect of pH variation on the bacteria communities and their electrochemical</p>

and biological answers, as preliminary study for sensing applications and hence, we did not look for strategy to improve the CE of our system.

13- How many times were repeated for polarization curve data? Error bar must be added.

At every data-point of characterization we performed polarization curve only once, simultaneously, in all the 9 MFCs. In the manuscript we showed a representative LSV for each triplicate this is why the error bar is not present. As an indication of the reproducibility in fig. 3c and in fig. 4a (for characterization of basic MFCs at day 48) we showed two different LSV obtained from two different MFCs within the triplicate. The values reported in the table are very similar. However, following the advice of the Reviewer we calculated the average of maximum power density value among the triplicate and standard deviation. The following table was added into SI section.

	Time (days)	pH _{feed}	pH _{anode} average	pH stdev	P _{max} (mW/m ²) average	P _{max} stdev
Neutral MFCs	16	7	7.2	0.05	27.7	2.8
	28	7	7.3	0.42	27	4.7
	48	7	7.9	0.23	24.2	4.2
triplicate	56	7	7.8	0.19	27.5	4.3
Basic MFCs	16	11	7.6	0.19	32.7	4.2
	28	11	7.5	0.09	33.3	8.1
	48	13	12.5	0.04	6.6	1.4
triplicate	56	7	10	0.13	57.5	5.4
Acidic MFCs	16	5	6.5	0.06	21.6	6.1
	28	5	6.4	0.23	20.2	3.9
	48	3	4.6	0.36	14.5	3.5
triplicate	56	7	5.2	0.21	16.1	3.2

14- The authors must be compare nobility of their work with other researchers work in this area.

The aim of our work is to analyze the adaptability of MFCs to slow and continuous variations of pH similar to those that could occur exposing the devices to unexpected, temporarily polluted fluid streams. For this reason we designed experiments able to mimic changes similar to those occurring in natural environments, running the MFCs in continuous mode, and modifying the pH values accordingly. In this approach lies the novelty of our work.

As highlighted in the Introduction, from line 47 to line 56, we discussed several works that are present in the literature in which the effect of pH variation is analyzed. None of them report an experimental approach similar to the one we used. Indeed batch or fed-batch mode are usually selected, working with fixed

	<p>pH values: with this approaches the adaptability of the biological system is usually not investigated.</p> <p>The key novelty of our work lies in the new experimental approach that we propose, that aims at designing pH changes as slow events occurring under continuous mode operation. We appreciate the comment of the Reviewer and changed the Introduction to highlight the novelty (from line 58 to line 60).</p>
<p>15- Because of using buffer solution in cathode compartment, basic condition in anode chamber is not suitable for proton to be able to transfer to cathode, because they prefer to be settled in anolyte compared to transfer to buffer catholyte.</p>	<p>We used buffered catholyte solution to avoid large pH variations able to influence MFC performance. This working condition, let us to associate the electrochemical performance observed to the anodic pH influent (Kaushik A. et al. 2013). In literature many works focused on the effect of anodic pH, reported the use of buffer in the catholyte solution. For i.e., Jia Q. et al. 2014, used a two chamber MFC where the cathode compartment was filled with a buffered solution having the same composition used in our tests (K₃Fe(CN)₆ 32.93 g/L; Na₂HPO₄ 20.7492 g/L and NaH₂PO₄ 3.1167 g/L). Raghavulu et al. 2009, performed the experiments at different anodic pH using two MFCs operated separately with K₃Fe(CN)₆ in phosphate buffer and aerated PBS. In order to investigate the role of anolyte pH on electroactive biofilm formation, Patil et al. 2011, utilized buffer solution in cathodic chamber, set to an equal pH-value as the anodic one. Furthermore, from a safety point of view, we decided to use a buffered catholyte due to the release of toxic hydrogen cyanide gas from Potassium Ferricyanide under acid conditions.</p> <p>Kaushik A, Chetal A. Power generation in microbial fuel cell fed with post methanation distillery effluent as a function of pH microenvironment. <i>Bioresource technology</i> 2013;147:77–83.</p> <p>Jia Q, Wei L, Han H, Shen J. Factors that influence the performance of two-chamber microbial fuel cell. <i>International Journal of Hydrogen Energy</i> 2014; 39:13687-13693.</p> <p>Raghavulu SV, Mohan SV, Goud RK, Sarma PN. Effect of anodic pH microenvironment on microbial fuel cell (MFC) performance in concurrence with aerated and ferricyanide catholytes. <i>Electrochemistry Communications</i> 2009;11:371–5.</p> <p>Patil SA, Harnisch F, Koch C, Hübschmann T, Fetzter I, Carmona-Martínez AA, et al. Electroactive mixed culture derived biofilms in microbial bioelectrochemical systems: the role of pH on biofilm formation, performance and composition. <i>Bioresource technology</i></p>

Reviewer 2**Evaluation of the manuscript: Effect of pH variations on anodic marine biofilm in a dual chamber microbial fuel cell**

In this article, the authors plan to test the behavior of an anodic electroactive biofilm established at neutral pH in the presence of acetate in response to drastic changes in pH (up to 3 and 13).

The experiments are conducted in MFC with two compartments (anolyte: synthetic seawater + acetate + peptone; catholyte: potassium ferricyanide + phosphate buffer, membrane: cation exchange membrane). The external resistance used was 2700 ohms.

The authors follow the pH of the anode compartment over time. Polarization curves (Voltage = f (current) and EIS analyzes are presented for different times. The authors also present as "supplementary figures" measures of COD or viability of cultivable bacteria at key moments of the experiments.

Finally, some bacterial communities particularly described in the biofilm anode are targeted and sought in the biofilms formed on the anodes.

1. Anodic microorganisms source: Why choose an inoculum taken from seawater at the interface between water and atmosphere if the objective is to form microbial anodes? This inoculum is susceptible to contain greatly aerobic communities. But the anodophilic communities are rather anaerobic. Also the communities targeted in the manuscript by rt-qPCR analyses are almost exclusively anaerobic communities (*Clostridium*, *Geobacter*, methanogens, ...).

The selection of the inoculum was influenced by the necessity to develop biosensing systems able to work at the interface between water and atmosphere. We have already used an inoculum sampled in the same way, and, as demonstrated in Tommasi et al., 2016, the anaerobic genera can be enriched in an MFC starting from such a sample. However, we recognize that the targeted genera have only a limited representation of the entire inoculum and that is why we are developing a community structure technique in order to better characterize the whole community of microorganisms that has grown inside the different MFC. Preliminary data confirmed the presence of both aerobic and facultative anaerobic bacteria belonging to Firmicutes and Proteobacteria Phylum.

Tommasi T, Sacco A., Armato C., Hidalgo D., Millone L, Sanginario A., Tresso E., Schilirò T., Pirri C.F., Dynamical analysis of microbial fuel cells based on planar and 3D packed Anodes, Chemical Engineering Journal (2016) 288: 38–49.

2. The authors claim the much applied nature of their study. But is it truly possible in marine open environments or in rivers, the pH, even local, undergoes changes as important as a Δ

As indicated in the text the pH chosen for the second phase of the experiment were extreme and poorly representative of natural occurring circumstances but they were necessary to introduce an effective change in the

pH of 6? The marine environment is well known for its resilience.

anodic chamber. In particular, the research performed wanted to evaluate the theoretical limits of recovery and adaptability of the MFC system. Moreover, for specific application such as biosensing the device has to be placed very closed to the water stream line and therefore a severe change in local pH cannot be excluded.

3. All experiments were performed in triplicate (as indicated by the authors). However reproducibility and the deviation of the results are not discussed in the manuscript.

Accordingly to the Reviewer's suggestion we revised the text indicating the variability within each triplicate. In particular, in the case of pH graph, shown in fig.2, the maximum variation in pH values at the same day within a triplicate was equal to 6.9% .

Considering, the variation of the voltage over time we have a maximum error within a triplicate that is equal to 16.2%.

In the case of polarization curve and impedance spectra one representative MFC among the triple is shown in the manuscript. As an indication of the reproducibility in fig. 3c and in fig. 4a (for characterization of basic MFCs at day 48) we shown two different LSV obtained from two different MFCs within the triplicate. The value reported in the table are very similar. Moreover, as reported in comment 13 Reviewer 1, to better clarify the reproducibility of the results we added the table below in SI, that shows the average of maximum power density within the triplicate and standard deviation.

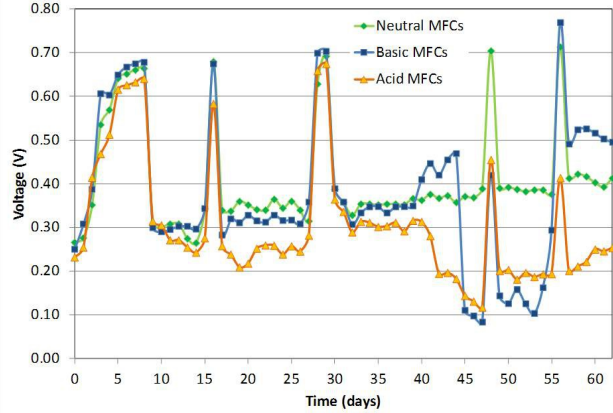
	Time (days)	pH _{anod}	pH _{anode} average	pH stdev	P _{max} (mW/m ²) average	P _{max} stdev
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	48	3	4.6	0.36	14.5	3.5
triplicate	56	7	5.2	0.21	16.1	3.2

4. The authors describe a "self-regulation" of the pH around neutrality in the anolyte, whatever the pH bias created upstream. An abiotic control with a sterilized inoculum did not present this "self-regulation" but did not furnish current also I guess. A complementary biotic control without additional electrode (without electrochemical phenomenon) could actually help to conclude that microbiology is primarily responsible for the observed phenomenon. The transport of ionic species,

In our laboratory experience running MFCs, with same design as described in the paper, but different inoculum source, results in an acidification of the anode. This finding is well represented in the literature where the acidification of the anode and the alkalization of the cathode are common results in long running MFC in the absence of a phosphate buffer (Zhuang L, et al. 2010; Gil, G.-C., et al. 2003; Oliveira VB, et al. 2013).

In particular, with the abiotic control we wanted to exclude the possible buffer effect due to the synthetic sea

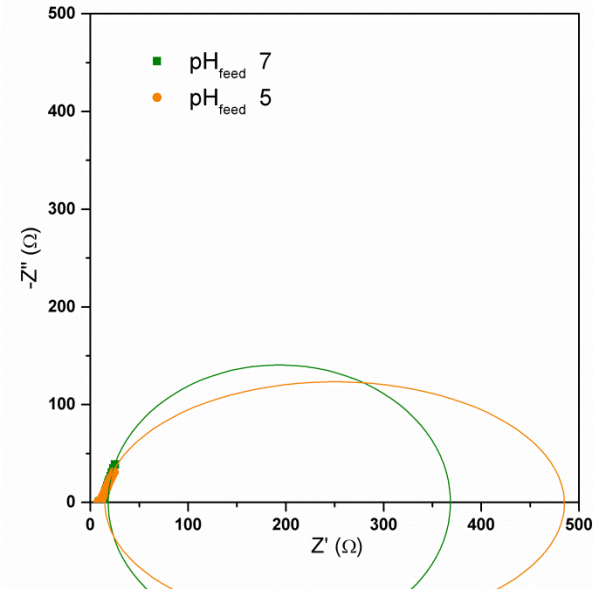
<p>especially protons, are accentuated in running MFC. Also species that migrate are different depending on the pH.</p>	<p>water and we explicated our intent into the manuscript.</p> <p>Moreover, in accordance to the interesting suggestion, we revised the text attributing self-regulating ability to the overall electrochemical system not solely to the biotic component.</p> <p>Zhuang L, Zhou S, Li Y, Yuan Y. Enhanced performance of air-cathode two-chamber microbial fuel cells with high-pH anode and low-pH cathode. <i>Bioresource technology</i> 2010;101:3514–9</p> <p>Gil, G.-C., Chang, I.-S., Kim, B.H., Kim, M., Jang, J.-K., Park, H.S., Kim, H.J., 2003. Operational parameters affecting the performance of a mediator-less microbial fuel cell. <i>Biosens. Bioelectron.</i> 18, 327–334.</p> <p>Oliveira VB, Simões M, Melo LF, Pinto AMFR. Overview on the developments of microbial fuel cells. <i>Biochemical Engineering Journal</i> 2013;73:53–64</p>
<p>5. The pH also has an important role on the bioavailability of the organic matter, especially organic acids (protonation, deprotonation, complexation, ...).</p>	<p>We certainly agree with the Reviewer about the important role of pH in making more available the organic matter for bacteria metabolism. However, since we are working in a “complex system”, where the complexity is increased by the use of a mixed consortium, it is quite difficult to attribute the effect we observed to a specific modification of the organic matter bioavailability instead of direct effect on microorganism growth. Moreover, using an organic matter already easy to be used by microorganisms, we have partially excluded the effect of hydrolysis that extreme pH can have, improving the bioavailability of complex organic matter (Ruggeri and Tommasi, 2012).</p> <p>Ruggeri, Tommasi. Efficiency and efficacy of pre-treatment and bioreaction for bio-H₂ energy production from organic waste. <i>International Journal of Hydrogen Energy</i>, 37 (8), 2012.</p>
<p>6. The authors speak several times about pH of the microenvironment referring here to the pH of the biofilm. However, the pH measurements are rather macroscopic, at the scale of the bulk. No pH measurement have been performed locally within the biofilm. Some theories rightly defend pH control mechanisms at the local level within the biofilm. But here nothing is sure ...</p>	<p>As correctly underlined, we have assumed that the macroscopic measurement in the bulk was representative of local modification. To avoid misunderstanding, we revised the text and talked more in general about the biotic component without discrimination between biofilm and planktonic of the bulk solution.</p>
<p>7. Plate count tests (material and methods): the agar growth medium is not adapted for marine bacteria growth. It is free of NaCl. On the fig. S2, no distinction is made between CFU obtained in aerobic condition from CFU obtained in anaerobic conditions. This technique is limited since the vast majority of marine bacteria are not cultivable, especially if limited to the heterotrophic metabolism.</p>	<p>Plate counts tests were performed using standard plate count agar (PCA) recipe dissolved in synthetic sea water to mimic marine environment. We revised the material and method which were not exhaustive on this point. Moreover, the fig. S2 was corrected to specify that the results shown are related to the aerobic condition. We agree on the limits of this technique due to the different requirements in growth conditions and presence of non-cultivable microorganism, as confirmed by DGGE preliminary data. However, this approach gave us a</p>

	<p>general indication on the number of viable microorganism. The CFU result together with the FESEM analysis of the biofilm suggested a correlation between the biotic development and the MFC performances.</p>																																																																
<p>Introduction: the terminology of “MFC” is not appropriately used. The authors seem to make no distinction between "bioelectrochemical systems" (BES) and "microbial fuel cells" (MFC)</p>	<p>The use of terminology “MFC” was checked and corrected.</p>																																																																
<p>Numbering pages and lines</p>	<p>Done.</p>																																																																
<p>No monitoring of voltage or current over time is presented</p>	<p>The graph representative of the average voltage within a triplicate over-time was added in SI and reported below. The first 9 days as well as the peaks at time-days 16, 28, 48, 56 correspond to OCV condition.</p>  <table border="1" data-bbox="852 808 1461 1218"> <caption>Approximate Voltage (V) over Time (days) for MFCs</caption> <thead> <tr> <th>Time (days)</th> <th>Neutral MFCs (V)</th> <th>Basic MFCs (V)</th> <th>Acid MFCs (V)</th> </tr> </thead> <tbody> <tr><td>0</td><td>0.25</td><td>0.25</td><td>0.25</td></tr> <tr><td>5</td><td>0.65</td><td>0.65</td><td>0.65</td></tr> <tr><td>10</td><td>0.68</td><td>0.68</td><td>0.68</td></tr> <tr><td>15</td><td>0.30</td><td>0.30</td><td>0.30</td></tr> <tr><td>20</td><td>0.35</td><td>0.35</td><td>0.35</td></tr> <tr><td>25</td><td>0.35</td><td>0.35</td><td>0.35</td></tr> <tr><td>30</td><td>0.70</td><td>0.70</td><td>0.70</td></tr> <tr><td>35</td><td>0.35</td><td>0.35</td><td>0.35</td></tr> <tr><td>40</td><td>0.35</td><td>0.35</td><td>0.35</td></tr> <tr><td>45</td><td>0.35</td><td>0.35</td><td>0.35</td></tr> <tr><td>48</td><td>0.70</td><td>0.70</td><td>0.70</td></tr> <tr><td>50</td><td>0.35</td><td>0.35</td><td>0.35</td></tr> <tr><td>55</td><td>0.35</td><td>0.35</td><td>0.35</td></tr> <tr><td>56</td><td>0.75</td><td>0.75</td><td>0.75</td></tr> <tr><td>60</td><td>0.40</td><td>0.40</td><td>0.40</td></tr> </tbody> </table>	Time (days)	Neutral MFCs (V)	Basic MFCs (V)	Acid MFCs (V)	0	0.25	0.25	0.25	5	0.65	0.65	0.65	10	0.68	0.68	0.68	15	0.30	0.30	0.30	20	0.35	0.35	0.35	25	0.35	0.35	0.35	30	0.70	0.70	0.70	35	0.35	0.35	0.35	40	0.35	0.35	0.35	45	0.35	0.35	0.35	48	0.70	0.70	0.70	50	0.35	0.35	0.35	55	0.35	0.35	0.35	56	0.75	0.75	0.75	60	0.40	0.40	0.40
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Reviewer 1

The manuscript entitle of " Effect of pH variations on anodic marine biofilm in a dual chamber microbial fuel cell " (Manuscript Number: HE_2016_1187)., by Valentina Margaria et al, depicts the effect of anodic pH on MFCs inoculated with marine consortia was investigated in order to define the pH extreme boundaries beyond which MFCs do not run properly, and to characterize the biofilm adaptation to possible pH environmental changes. Before the manuscript deems acceptable for publication, it needs be revised very well. Some of specific suggestions are listed below:

1- The authors must be rechecking the English of the manuscript. It is fairly poor and must me improvement before publication by native person.	Accordingly, the manuscript was rechecked by a native English speaker
2- The authors must be more highlight in abstract section.	The abstract section was revised
3- What means the final sentences of abstract section? " biosensor for on-site seawater monitoring or power supply for remote area"	The final sentence in the abstract section was reformulated to clarify the meaning.
4- Why the inoculum was taken at the interface between water and atmosphere?	<p>The choice of inoculum taken at the interface between seawater and atmosphere was made in order to see how a resident population tolerant to oxigen (all bacteria, and not specific ones) can electrochemically behave inside an MFC. In particular, we are involved in projects where MFCs power either electronics or sensors integrated into autonomous underwater vehicle that can operate at the sea surface as well as at different depth. For this reason, it was interesting to investigate the behavior of the selected inoculum into the MFCs. Moreover, this was not the first time that we used an inoculum sampled in the same way: in Tommasi et al., 2016 the presence and the enrichment of electrogenic microorganisms, such as <i>Shewanella</i> and <i>Geobacter</i>, was demonstrated.</p> <p>Tommasi T. Sacco A., Armato C., Hidalgo D., Millone L, Sanginario A., Tresso E., Schilirò T., Pirri C.F., Dynamical analysis of microbial fuel cells based on planar and 3D packed Anodes, Chemical Engineering Journal (2016) 288: 38–49.</p>
5- The authors used mixed culture in their study. Why they selected sterile conditions?	The tests were carried out under sterile condition to avoid cross-contamination from different environmental samples used in our laboratory. Moreover, operating in this sterile condition, let us to attribute electrochemical performances to the starting mixed culture.
6- The authors talked about bacterial growth in material and method section but they did not	As described in section 2.1, we estimated bacterial growth measuring the Optical Density at 600 nm (OD ₆₀₀) during the inoculum enrichment steps. OD ₆₀₀

<p>present or talk about it in their manuscript at all.</p>	<p>was determined to identify the optimal growth-phase for the microbial sub-culture passage. No measures were performed on samples taken from working MFCs. We improved the description in section 2.1 to avoid any confusion.</p>
<p>7- What was the HRT for cathode chamber for ferricyanide.</p>	<p>We chose a catholyte recirculating system using a peristaltic pump to ensure a stable cathodic performance. The catholyte was placed in a 500 ml glass bottle as reservoir and recirculated through the cathodic chamber at a speed of 40 rpm. This operational mode included the replacement of the recirculating catholyte solution every 72 h.</p>
<p>8- From the figure 3 b, it can be understand the pH 5 has in contrary with data provide inset.</p>	<p>As it is reported in the figure here below, the resistance values are strictly related to the slope of the Nyquist arcs, in the sense that an arc whose initial slope is steeper (green curve in the figure below) will lead to a resistance which would be lower with respect to an arc with lower steepness (orange curve), no matter of the $-Z''$ values at a certain frequency (in Fig. 3b the last points of all the three curves were acquired at 100 mHz), which is instead related to the capacitance behavior. For this reason, being the pH5 curve characterized by lower steepness with respect to the other two curves shown in Fig. 3b in the manuscript, its resistance should be larger, and this is in accordance with the values obtained by the fitting procedure, as reported in the table (Inset in Fig. 3b).</p> 
<p>9- Figure 1 is not informative and must be improve</p>	<p>We revised the Figure 1 adding a brief description of each phase to clarify the experimental process.</p>
<p>10- The authors must be provide EIS circuit mode in the figure of EIS spectra as an inset.</p>	<p>The equivalent electrical circuit used for the fitting of the impedance spectra was added as an inset in Fig.</p>

	3b. The figure caption was modified accordingly.																																																																																					
11- Schematic and photograph image of MFC is necessary in supplementary material section.	A schematic of the MFC and recirculating system as well as photographs were added in the supporting information.																																																																																					
12- One of important parameter on MFC performances is columbic efficiency.	According to the Reviewer's comment, we have calculated Coulombic efficiency (CE), that results in all case very low (<1%). We associated this low value mainly to the high external load used during the test that brings to low current recovery, considering that we were working near Open Circuit Condition. Even if low, the trend reflects the behavior of acid, basic and neutral MFCs, that is that acid MFCs have worse performances than neutral and basic MFCs. However, the aim of our work was mainly focused on the effect of pH variation on the bacteria communities and their electrochemical and biological answers, as preliminary study for sensing applications and hence, we did not look for strategy to improve the CE of our system.																																																																																					
13- How many times were repeated for polarization curve data? Error bar must be added.	<p>At every data-point of characterization we performed polarization curve only once, simultaneously, in all the 9 MFCs. In the manuscript we showed a representative LSV for each triplicate this is why the error bar is not present. As an indication of the reproducibility in fig. 3c and in fig. 4a (for characterization of basic MFCs at day 48) we showed two different LSV obtained from two different MFCs within the triplicate. The values reported in the table are very similar. However, following the advice of the Reviewer we calculated the average of maximum power density value among the triplicate and standard deviation. The following table was added into SI section.</p> <table border="1"> <thead> <tr> <th></th> <th>Time (days)</th> <th>pH_{feed}</th> <th>pH_{anode} average</th> <th>pH stdev</th> <th>P_{max} (mW/m²) average</th> <th>P_{max} stdev</th> </tr> </thead> <tbody> <tr> <td rowspan="3">Neutral MFCs</td> <td>16</td> <td>7</td> <td>7.2</td> <td>0.05</td> <td>27.7</td> <td>2.8</td> </tr> <tr> <td>28</td> <td>7</td> <td>7.3</td> <td>0.42</td> <td>27</td> <td>4.7</td> </tr> <tr> <td>48</td> <td>7</td> <td>7.9</td> <td>0.23</td> <td>24.2</td> <td>4.2</td> </tr> <tr> <td>triplicate</td> <td>56</td> <td>7</td> <td>7.8</td> <td>0.19</td> <td>27.5</td> <td>4.3</td> </tr> <tr> <td rowspan="3">Basic MFCs</td> <td>16</td> <td>11</td> <td>7.6</td> <td>0.19</td> <td>32.7</td> <td>4.2</td> </tr> <tr> <td>28</td> <td>11</td> <td>7.5</td> <td>0.09</td> <td>33.3</td> <td>8.1</td> </tr> <tr> <td>48</td> <td>13</td> <td>12.5</td> <td>0.04</td> <td>6.6</td> <td>1.4</td> </tr> <tr> <td>triplicate</td> <td>56</td> <td>7</td> <td>10</td> <td>0.13</td> <td>57.5</td> <td>5.4</td> </tr> <tr> <td rowspan="3">Acidic MFCs</td> <td>16</td> <td>5</td> <td>6.5</td> <td>0.06</td> <td>21.6</td> <td>6.1</td> </tr> <tr> <td>28</td> <td>5</td> <td>6.4</td> <td>0.23</td> <td>20.2</td> <td>3.9</td> </tr> <tr> <td>48</td> <td>3</td> <td>4.6</td> <td>0.36</td> <td>14.5</td> <td>3.5</td> </tr> <tr> <td>triplicate</td> <td>56</td> <td>7</td> <td>5.2</td> <td>0.21</td> <td>16.1</td> <td>3.2</td> </tr> </tbody> </table>		Time (days)	pH _{feed}	pH _{anode} average	pH stdev	P _{max} (mW/m ²) average	P _{max} stdev	Neutral MFCs	16	7	7.2	0.05	27.7	2.8	28	7	7.3	0.42	27	4.7	48	7	7.9	0.23	24.2	4.2	triplicate	56	7	7.8	0.19	27.5	4.3	Basic MFCs	16	11	7.6	0.19	32.7	4.2	28	11	7.5	0.09	33.3	8.1	48	13	12.5	0.04	6.6	1.4	triplicate	56	7	10	0.13	57.5	5.4	Acidic MFCs	16	5	6.5	0.06	21.6	6.1	28	5	6.4	0.23	20.2	3.9	48	3	4.6	0.36	14.5	3.5	triplicate	56	7	5.2	0.21	16.1	3.2
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14- The authors must be compare nobility of their work with other researchers work in this area.	The aim of our work is to analyze the adaptability of MFCs to slow and continuous variations of pH similar to those that could occur exposing the devices to unexpected, temporarily polluted fluid streams. For this reason we designed experiments able to mimic changes similar to those occurring in natural environments, running the MFCs in continuous mode, and modifying the pH values accordingly. In this approach lies the																																																																																					

	<p>novelty of our work.</p> <p>As highlighted in the Introduction, from line 47 to line 56, we discussed several works that are present in the literature in which the effect of pH variation is analyzed. None of them report an experimental approach similar to the one we used. Indeed batch or fed-batch mode are usually selected, working with fixed pH values: with this approaches the adaptability of the biological system is usually not investigated.</p> <p>The key novelty of our work lies in the new experimental approach that we propose, that aims at designing pH changes as slow events occurring under continuous mode operation. We appreciate the comment of the Reviewer and changed the Introduction to highlight the novelty (from line 58 to line 60).</p>
<p>15- Because of using buffer solution in cathode compartment, basic condition in anode chamber is not suitable for proton to be able to transfer to cathode, because they prefer to be settled in anolyte compared to transfer to buffer catholyte.</p>	<p>We used buffered catholyte solution to avoid large pH variations able to influence MFC performance. This working condition, let us to associate the electrochemical performance observed to the anodic pH influent (Kaushik A. et al. 2013). In literature many works focused on the effect of anodic pH, reported the use of buffer in the catholyte solution. For i.e., Jia Q. et al. 2014, used a two chamber MFC where the cathode compartment was filled with a buffered solution having the same composition used in our tests (K₃Fe(CN)₆ 32.93 g/L; Na₂HPO₄ 20.7492 g/L and NaH₂PO₄ 3.1167 g/L). Raghavulu et al. 2009, performed the experiments at different anodic pH using two MFCs operated separately with K₃Fe(CN)₆ in phosphate buffer and aerated PBS. In order to investigate the role of anolyte pH on electroactive biofilm formation, Patil et al. 2011, utilized buffer solution in cathodic chamber, set to an equal pH-value as the anodic one. Furthermore, from a safety point of view, we decided to use a buffered catholyte due to the release of toxic hydrogen cyanide gas from Potassium Ferricyanide under acid conditions.</p> <p>Kaushik A, Chetal A. Power generation in microbial fuel cell fed with post methanation distillery effluent as a function of pH microenvironment. <i>Bioresource technology</i> 2013;147:77–83.</p> <p>Jia Q, Wei L, Han H, Shen J. Factors that influence the performance of two-chamber microbial fuel cell. <i>International Journal of Hydrogen Energy</i> 2014; 39:13687-13693.</p> <p>Raghavulu SV, Mohan SV, Goud RK, Sarma PN. Effect of anodic pH microenvironment on microbial fuel cell (MFC) performance in concurrence with aerated and ferricyanide catholytes. <i>Electrochemistry Communications</i> 2009;11:371–5.</p> <p>Patil SA, Harnisch F, Koch C, Hübschmann T, Fetzter I, Carmona-Martínez AA, et al. Electroactive mixed culture derived biofilms in microbial bioelectrochemical systems: the role of pH on biofilm formation, performance and composition. <i>Bioresource technology</i> 2011;102:9683–90.</p>

Reviewer 2

Evaluation of the manuscript: Effect of pH variations on anodic marine biofilm in a dual chamber microbial fuel cell

In this article, the authors plan to test the behavior of an anodic electroactive biofilm established at neutral pH in the presence of acetate in response to drastic changes in pH (up to 3 and 13).

The experiments are conducted in MFC with two compartments (anolyte: synthetic seawater + acetate + peptone; catholyte: potassium ferricyanide + phosphate buffer, membrane: cation exchange membrane). The external resistance used was 2700 ohms.

The authors follow the pH of the anode compartment over time. Polarization curves (Voltage = f (current) and EIS analyzes are presented for different times. The authors also present as "supplementary figures" measures of COD or viability of cultivable bacteria at key moments of the experiments.

Finally, some bacterial communities particularly described in the biofilm anode are targeted and sought in the biofilms formed on the anodes.

1. Anodic microorganisms source: Why choose an inoculum taken from seawater at the interface between water and atmosphere if the objective is to form microbial anodes? This inoculum is susceptible to contain greatly aerobic communities. But the anodophilic communities are rather anaerobic. Also the communities targeted in the manuscript by rt-qPCR analyses are almost exclusively anaerobic communities (*Clostridium*, *Geobacter*, methanogens, ...).

The selection of the inoculum was influenced by the necessity to develop biosensing systems able to work at the interface between water and atmosphere. We have already used an inoculum sampled in the same way, and, as demonstrated in Tommasi et al., 2016, the anaerobic genera can be enriched in an MFC starting from such a sample. However, we recognize that the targeted genera have only a limited representation of the entire inoculum and that is why we are developing a community structure technique in order to better characterize the whole community of microorganisms that has grown inside the different MFC. Preliminary data confirmed the presence of both aerobic and facultative anaerobic bacteria belonging to Firmicutes and Proteobacteria Phylum.

Tommasi T. Sacco A., Armato C., Hidalgo D., Millone L., Sanginario A., Tresso E., Schilirò T., Pirri C.F., Dynamical analysis of microbial fuel cells based on planar and 3D packed Anodes, Chemical Engineering Journal (2016) 288: 38–49.

2. The authors claim the much applied nature of their study. But is it truly possible in marine open environments or in rivers, the pH, even local, undergoes changes as important as a Δ pH of 6? The marine environment is well known for its resilience.

As indicated in the text the pH chosen for the second phase of the experiment were extreme and poorly representative of natural occurring circumstances but they were necessary to introduce an effective change in the anodic chamber. In particular, the research performed wanted to evaluate the theoretical limits of recovery and adaptability of the MFC system. Moreover, for specific application such as biosensing the device has to be placed very closed to the water stream line and therefore a severe change in local pH cannot be excluded.

3. All experiments were performed in triplicate (as indicated by the authors). However reproducibility and the deviation of the results are not discussed in the manuscript.

Accordingly to the Reviewer's suggestion we revised the text indicating the variability within each triplicate. In particular, in the case of pH graph, shown in fig.2, the maximum variation in pH values at the same day within a triplicate was equal to 6.9% .

Considering, the variation of the voltage over time we have a maximum error within a triplicate that is equal to 16.2%.

In the case of polarization curve and impedance

	<p>spectra one representative MFC among the triple is shown in the manuscript. As an indication of the reproducibility in fig. 3c and in fig. 4a (for characterization of basic MFCs at day 48) we shown two different LSV obtained from two different MFCs within the triplicate. The value reported in the table are very similar. Moreover, as reported in comment 13 Reviewer 1, to better clarify the reproducibility of the results we added the table below in SI, that shows the average of maximum power density within the triplicate and standard deviation.</p> <table border="1" data-bbox="807 551 1426 869"> <thead> <tr> <th></th> <th>Time (days)</th> <th>pH_{fluid}</th> <th>pH_{anode} average</th> <th>pH stdev</th> <th>P_{max} (mW/m²) average</th> <th>P_{max} stdev</th> </tr> </thead> <tbody> <tr> <td rowspan="3">Neutral MFCs</td> <td>16</td> <td>7</td> <td>7.2</td> <td>0.05</td> <td>27.7</td> <td>2.8</td> </tr> <tr> <td>28</td> <td>7</td> <td>7.3</td> <td>0.42</td> <td>27</td> <td>4.7</td> </tr> <tr> <td>48</td> <td>7</td> <td>7.9</td> <td>0.23</td> <td>24.2</td> <td>4.2</td> </tr> <tr> <td>triplicate</td> <td>56</td> <td>7</td> <td>7.8</td> <td>0.19</td> <td>27.5</td> <td>4.3</td> </tr> <tr> <td rowspan="3">Basic MFCs</td> <td>16</td> <td>11</td> <td>7.6</td> <td>0.19</td> <td>32.7</td> <td>4.2</td> </tr> <tr> <td>28</td> <td>11</td> <td>7.5</td> <td>0.09</td> <td>33.3</td> <td>8.1</td> </tr> <tr> <td>48</td> <td>13</td> <td>12.5</td> <td>0.04</td> <td>6.6</td> <td>1.4</td> </tr> <tr> <td>triplicate</td> <td>56</td> <td>7</td> <td>10</td> <td>0.13</td> <td>57.5</td> <td>5.4</td> </tr> <tr> <td rowspan="3">Acidic MFCs</td> <td>16</td> <td>5</td> <td>6.5</td> <td>0.06</td> <td>21.6</td> <td>6.1</td> </tr> <tr> <td>28</td> <td>5</td> <td>6.4</td> <td>0.23</td> <td>20.2</td> <td>3.9</td> </tr> <tr> <td>48</td> <td>3</td> <td>4.6</td> <td>0.36</td> <td>14.5</td> <td>3.5</td> </tr> <tr> <td>triplicate</td> <td>56</td> <td>7</td> <td>5.2</td> <td>0.21</td> <td>16.1</td> <td>3.2</td> </tr> </tbody> </table>		Time (days)	pH _{fluid}	pH _{anode} average	pH stdev	P _{max} (mW/m ²) average	P _{max} stdev	Neutral MFCs	16	7	7.2	0.05	27.7	2.8	28	7	7.3	0.42	27	4.7	48	7	7.9	0.23	24.2	4.2	triplicate	56	7	7.8	0.19	27.5	4.3	Basic MFCs	16	11	7.6	0.19	32.7	4.2	28	11	7.5	0.09	33.3	8.1	48	13	12.5	0.04	6.6	1.4	triplicate	56	7	10	0.13	57.5	5.4	Acidic MFCs	16	5	6.5	0.06	21.6	6.1	28	5	6.4	0.23	20.2	3.9	48	3	4.6	0.36	14.5	3.5	triplicate	56	7	5.2	0.21	16.1	3.2
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<p>4. The authors describe a "self-regulation" of the pH around neutrality in the anolyte, whatever the pH bias created upstream. An abiotic control with a sterilized inoculum did not present this "self-regulation" but did not furnish current also I guess. A complementary biotic control without additional electrode (without electrochemical phenomenon) could actually help to conclude that microbiology is primarily responsible for the observed phenomenon. The transport of ionic species, especially protons, are accentuated in running MFC. Also species that migrate are different depending on the pH.</p>	<p>In our laboratory experience running MFCs, with same design as described in the paper, but different inoculum source, results in an acidification of the anode. This finding is well represented in the literature where the acidification of the anode and the alkalization of the cathode are common results in long running MFC in the absence of a phosphate buffer (Zhuang L, et al. 2010; Gil, G.-C., et al. 2003; Oliveira VB, et al. 2013).</p> <p>In particular, with the abiotic control we wanted to exclude the possible buffer effect due to the synthetic sea water and we explicated our intent into the manuscript.</p> <p>Moreover, in accordance to the interesting suggestion, we revised the text attributing self-regulating ability to the overall electrochemical system not solely to the biotic component.</p> <p>Zhuang L, Zhou S, Li Y, Yuan Y. Enhanced performance of air-cathode two-chamber microbial fuel cells with high-pH anode and low-pH cathode. <i>Bioresource technology</i> 2010;101:3514-9</p> <p>Gil, G.-C., Chang, I.-S., Kim, B.H., Kim, M., Jang, J.-K., Park, H.S., Kim, H.J., 2003. Operational parameters affecting the performance of a mediator-less microbial fuel cell. <i>Biosens. Bioelectron.</i> 18, 327-334.</p> <p>Oliveira VB, Simões M, Melo LF, Pinto AMFR. Overview on the developments of microbial fuel cells. <i>Biochemical Engineering Journal</i> 2013;73:53-64</p>																																																																																					
<p>5. The pH also has an important role on the bioavailability of the organic matter!, especially organic acids (protonation, deprotonation, complexation, ...).</p>	<p>We certainly agree with the Reviewer about the important role of pH in making more available the organic matter for bacteria metabolism. However, since we are working in a "complex system", where the complexity is increased by the use of a mixed consortium, it is quite difficult to attribute the effect we observed to a specific modification of the organic matter bioavailability instead of direct effect on microorganism growth. Moreover, using an organic matter already easy to be used by microorganisms, we have partially excluded the effect of hydrolysis that extreme pH can have, improving the bioavailability of complex organic matter (Ruggeri and Tommasi, 2012).</p>																																																																																					

	<p>Ruggeri, Tommasi. Efficiency and efficacy of pre-treatment and bioreaction for bio-H₂ energy production from organic waste. <i>International Journal of Hydrogen Energy</i>, 37 (8), 2012.</p>
<p>6. The authors speak several times about pH of the microenvironment referring here to the pH of the biofilm. However, the pH measurements are rather macroscopic, at the scale of the bulk. No pH measurement have been performed locally within the biofilm. Some theories rightly defend pH control mechanisms at the local level within the biofilm. But here nothing is sure ...</p>	<p>As correctly underlined, we have assumed that the macroscopic measurement in the bulk was representative of local modification. To avoid misunderstanding, we revised the text and talked more in general about the biotic component without discrimination between biofilm and planktonic of the bulk solution.</p>
<p>7. Plate count tests (material and methods): the agar growth medium is not adapted for marine bacteria growth. It is free of NaCl. On the fig. S2, no distinction is made between CFU obtained in aerobic condition from CFU obtained in anaerobic conditions. This technique is limited since the vast majority of marine bacteria are not cultivable, especially if limited to the heterotrophic metabolism.</p>	<p>Plate counts tests were performed using standard plate count agar (PCA) recipe dissolved in synthetic sea water to mimic marine environment. We revised the material and method which were not exhaustive on this point. Moreover, the fig. S2 was corrected to specify that the results shown are related to the aerobic condition. We agree on the limits of this technique due to the different requirements in growth conditions and presence of non-cultivable microorganism, as confirmed by DGGE preliminary data. However, this approach gave us a general indication on the number of viable microorganism. The CFU result together with the FESEM analysis of the biofilm suggested a correlation between the biotic development and the MFC performances.</p>
<p>Introduction: the terminology of "MFC" is not appropriately used. The authors seem to make no distinction between "bioelectrochemical systems" (BES) and "microbial fuel cells" (MFC)</p>	<p>The use of terminology "MFC" was checked and corrected.</p>
<p>Numbering pages and lines</p>	<p>Done.</p>
<p>No monitoring of voltage or current over time is presented</p>	<p>The graph representative of the average voltage within a triplicate over-time was added in SI and reported below. The first 9 days as well as the peaks at time-days 16, 28, 48, 56 correspond to OCV condition.</p> 