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Exploring natural vs. synthetic minimal media to boost current generation with electrochemically-active marine bioanodes

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ABSTRACT

One of the greatest challenges in the development of Microbial Electrochemical Technologies (METs) is the achievement of efficient bioanodes, which not only can operate for long term but which can also be effectively and promptly regenerated for the sustained and successive production of electricity out of waste organics contained in aqueous streams. Simple strategies that facilitate the engineering of these systems are then pursued. Sustainable electricity generation was here achieved using electrochemically-active marine biofilms, which reached up to 6.8 A m^{-2} in the best case. These biofilms showed deteriorated current generation after successive transfers in fresh natural media. The electricity-generation functionality of these marine biofilms was recuperated after their relocation into synthetic minimal media (i.e. up to about 3.8 A m^{-2} after a decay down to about $1\text{--}2 \text{ A m}^{-2}$). Upon this relocation, the overall electrochemical mechanisms were preserved. Fluctuating nutrient stress intensified the effect of minimal media on current generation. The change from natural to minimal media showed an important impact on the selection and adaptation of microbial communities, characterized by CE-SSCP profiles; yet, robust bioanodes in which some microbial species were preserved were obtained in synthetic minimal media, these being sufficient for a reproducible electrochemical functionality. The systematic cycling between natural media and minimal media, regarded as periodic stress conditioning, is therefore proposed as a convenient strategy to boost current generation in robust electrochemically-active marine bioanodes.

Keywords:

Microbial electrochemical technologies
Microbial fuel cells
Bioanodes
Electrochemically-active bacteria
Marine biofilms

1. Introduction

Microbial Electrochemical Technologies (METs) use microbial biofilms which develop over the surface of electrodes and act as catalysts for electrochemical oxidation or reduction reactions [1]. Such type of microbial electrocatalysis allows a broad range of applications when operating in galvanic (power generating) or electrolytic (power consuming) mode, for instance, electricity generation [2], treatment of low strength [3] and high strength wastewaters [4], water desalination [5], and electrosynthesis of organic chemicals [6], among others. Although some of these systems are still in early development—particularly for microbial electrosynthesis—they promise significant breakthroughs for the

valorisation waste streams into a selection of higher value chemicals and fuels [7–9].

The crucial component of some METs is the microbial anode. The source of inoculum for its development is considered to be one of the most influential factors on their performance. Numerous sources have been already proposed, including activated sludge [10], various wastewaters and mixtures thereof [11–17], and freshwater or its sediments [18–20].

Particularly, saline environments provide the opportunity to develop microbial anodes that operate at high ionic strength [21–23]. Such type of electrolytes enhance the transport of ions in the bulk liquid [24], which as a consequence reduces the ohmic drop [25,26] or ionic resistance of the electrolyte. In fact, media with high ionic conductivity are appropriate for better performance of any electrochemical system. However, in spite of the evident advantages for better electrochemical performance, the development of microbial anodes in highly conductive environments ($>50 \text{ mS/cm}$) is still on early stages. The majority of electrochemically-active (EA) bacteria revealed so far would be drastically affected by the changes in osmotic pressure that media with high

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ionic strength (see salinity) would entail. Yet, there exist environments where halotolerant microbial communities systematically face this challenge. Saltwater or its sediments have been employed showing modest performances on current generation [27–29]. Air-tolerant marine biofilms have been employed in microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) showing suitable performance and reproducibility [30,31]. Marine sediments [32–36] and in lesser extent salt marsh sediments [37–41] have also been tested as inoculum sources in sediment-MFCs. Although the latter inhibit the prevalence of methanogenic bacteria which are detrimental for electricity generation in MFCs and some cases have favourably showed to generate up to 85 A m^{-2} [42], the sediment fraction seems so far an essential constituent for long term operation, which results inconvenient for engineered systems. Handling the solid fraction is simply not practical for industrial prospection of METs.

Likewise, nutritional conditions represent a medullar aspect in the performance of METs. Direct feed of natural media [43–45], wastewaters containing diverse substrate organics [46–48] and other types of wastes [49,50], supplemented or not with particular nutriments, have been extensively explored.

Although highly desirable from the perspective of valorising or improving waste streams, the utilisation of such media might appear impractical from certain perspectives. From the scientific point of view, their complex composition obscures the understanding some fundamental phenomena and extracellular electron transfer mechanisms in microbial communities; they involve unidentified side reactions occurring at the bulk phase, adsorption of organic compounds and other events masking the electrochemical influence of the biofilm. The electrochemical activity of the biofilms may involve direct electron transfer via multi-heme cytochromes, electron transfer through nanowires, and indirect transfer via soluble mediators or electron shuttles. Simpler media would allow a better demonstration of such occurrences; at the same time, these would make easier to address the effect of certain operational parameters and subsequently to better regulate them. From the engineering perspective, other concerns arise. Not all types of usable industrial wastewaters contain significant organic loads. For instance, desalination processes waters typically undergo through a step of reduction of the total dissolved solids (TDS) content; seawater desalination starts with 35–45 g/L TDS, while brackish water starts with 1.5–15 g/L TDS, and they are reduced to below 0.5 g/L TDS. Therefore, complete culture media are seldom representative for conducting studies with true applicability.

Altogether this has further consequences, microbial consortia will develop at different levels of community and metabolic complexity [51]; simpler culture media influence the development of microbial consortia with simpler nutritional requirements, which are known to become more economical and easier to control at process level. Yet, this simpler metabolic complexity does not necessarily imply a less diverse microbial community. Besides, growth in defined media may result in increased biofilm formation compared to rich media [52]. Furthermore, from the perspective of microbial electrosynthesis (MES), well controlled and simpler matrices appear pragmatic for a subsequent downstream processing strategy—on the perspective to obtain high purity products or easier, faster and more economical recovery thereof.

Minimal culture media are those which only contain the critical nutrients for microbial growth: a carbon source, and various salts that provide essential elements such as nitrogen, phosphorous, magnesium, sulphur and other trace elements. Some of them can also be supplemented by selected agents, usually amino acids, vitamins or sugars, when the growth of auxotrophs is anticipated. Minimal media meet better the environmental conditions of surface-waters than complete media, reason why they are often

used in environmental microbiology to grow wild-type microorganisms [53]. Minimal media have been generally employed in MFCs with pure cultures of *Shewanella oneidensis*, *Lactococcus lactis*, *Enterobacter cloacae* and *Bacillus subtilis* [54–64]. Nevertheless, mixed culture bioanodes have predominantly derived from microbial communities enriched from wastewaters, and reports on their direct adaptation to minimal media are scarce [65]. What is more, such approach has not been reported for halotolerant bioelectrodes; knowledge is still lacking on the performance of electrochemically-active mixed culture bioanodes under minimal conditions in contrast to natural or industrial streams.

Based on the overall considerations here explained, the aim of the present investigation was to use halotolerant communities to develop EA-biofilms in minimal synthetic media, which were hypothesized to progress into robust, reproducible and electrochemically functional microbial anodes. Microbial anodes were formed from marine biofilms and their functional adaptation to minimal culture media was assessed. The present study provides significant advancement of the existing state of the art for its direct implications towards development and maintenance of robust bioelectrodes with industrial/engineered prospection.

2. Materials and methods

2.1. Biofilm samples

Natural marine biofilms were collected from a plastic floating bridge in the port of La Tremblade (N45.46'10", W1.8'30"; Atlantic Ocean, France), as previously described by Erable and Bergel [66]. The floating bridge is situated in the low water zone and, consequently, it is in direct contact with sediments for about 6 h a day. The biofilm was harvested using a plastic scraper; around 50 mL of biomaterial were stored in a glass bottle containing 20 mL of fresh seawater, for three days at ambient temperature ($17 \pm 4^\circ\text{C}$). The seawater used for the experiments was from the same location and it had a conductivity of 49 mS cm^{-1} . A biofilm mix was prepared by adding 1:1 of natural seawater and solid biofilm, supplemented with 20 mM sodium acetate, and taken to homogeneity in a ceramic mortar. The biofilm mix was maintained for 8 h under orbital agitation at 60 rpm, until use.

2.2. Electrochemical instrumentation and setup

Experiments were carried out in 700 mL (total volume) borosilicate Schott Duran reactors containing 500 mL of culture medium solution, at constant polarisation conditions (chronoamperometry, CA) using a conventional three-electrode system connected to a multi-potentiostat (VMP2 Bio-Logic SA, software EC-Lab v.9.97, Bio-Logic SA). The reactors were closed hermetically, with no gas flow. The operational temperature was controlled at 30°C throughout all the investigation. The experiments were conducted in a half-cell setup to evaluate the performance of each anode in well-controlled electrochemical conditions. Rectangular slices of 2 types of carbon electrodes were used as working electrodes, felt or tissue [67], respectively. During punctual points of experimentation, samples of the electrodes were taken with sharp scissors; therefore, current densities (A m^{-2}) are reported with the proper corrections to the projected surface area. The carbon materials were initially sterile, as proven by confocal microscopy (data not shown), and they were vigorously rinsed in sterile distilled water prior to use. The electrodes were embedded down to half of the liquid volume. The electric contact was made with twirled 90%/10% Platinum/Iridium wires. The auxiliary electrodes were made of Heraeus Vectra 90%/10% Platinum/Iridium meshes (20 cm^2 projected surface area) and were maintained face to face respect to the working electrode. All

electric potentials are here reported against the Saturated Calomel standard reference Electrode (SCE, Materials Mates, in saturated KCl which has a potential of 244 mV vs. SHE). The potential of the anodes was fixed at +100 mV/SCE during CA. From time to time the constant polarization was interrupted and cyclic voltammetry (CV) was instead performed *in situ* on the active anodes, starting from open circuit conditions. The potential was scanned three times between -500 and 500 mV/SCE at scan rate of 5 mV s⁻¹; only data from the last scan (out of three) are here reported. Control electrodes (seawater or synthetic culture media without inoculum) were equally tested, showing no electric current generation.

2.3. Media and culture conditions

Formation of the EA-biofilms was achieved by introducing the inoculum (10% v/v) into a natural medium (NM) or directly in a synthetic minimal medium. NM was composed of fresh seawater supplemented with acetate. For each independent experiment, the microbial anode was then repetitively transferred into a synthetic minimal medium (SMM). Bioanode transfer to fresh media was carried out once that a peak of current had decayed, as per observations on the response in CA. The SMM consisted of a modified Starkey medium [68], frequently used for culturing strict anaerobic sulfate-reducing bacteria. The composition of this medium was, per liter of distilled water: K₂HPO₄ 0.5 g, NH₄Cl₂ g, oligoelements solution 1 mL and NaCl 30 g. The media can be supplemented with yeast extract yeast extract (0.2 g), in which case it is here referred as SMM-Y. The oligoelements solution was composed of, per liter of distilled water: HCl 37% 46 mL, MgCl₂·6H₂O 55 g, FeSO₄(NH₄)₂SO₄·6H₂O 7 g, ZnCl₂·2H₂O 1 g, MnCl₂·4H₂O 1.2 g, CuSO₄·5H₂O 0.4 g, CoSO₄·7H₂O 1.3 g, BO₃H₃, 1 g Mo₇O₂₄(NH₄)₆·4H₂O, 0.05 g NiCl₂·6H₂O and Na₂SeO₃·5H₂O 0.01 g; these compounds were dissolved under stirring at 200 rpm during one hour, then the solution was heated up until complete dissolution of the solid salts. After cooling under agitation, 60 g of CaCl₂·2H₂O were added. The solution was stocked in dark flask under refrigeration; it precipitates after several days, so it needs to be re-homogenized prior to utilization. The media in the reactors were supplemented with sodium acetate. Here, the concentration of sodium acetate (either in NM or SMM) is marked as a subindex in mM (e.g. SMM₂₀ refers to a SMM medium supplemented with 20 sodium acetate; NM₆₀ refers to the natural medium supplemented with 60 mM sodium acetate, and so on). The conductivity of each medium was measured within 48 and 52 mS cm⁻¹, the temperature was kept constant at 30 °C and the systems had no agitation. The pH was naturally maintained (buffered) between 7.5 and 8.2 during all the experiments.

2.4. DNA extraction, CE-SSCP fingerprinting and DNA sequencing

Genomic DNA was extracted and purified from the carbon electrode samples using a previously described protocol [69]. The total DNA extracted was purified using a QiAmp DNA microkit (Qiagen, Hilden, Germany). DNA amount and purity of extracts were confirmed by spectrophotometry (Infinite NanoQuant M200, Tecan, Austria). The bacterial communities issued of the EA-biofilms were analyzed by the PCR-single strand conformation polymorphism (SSCP) technique. Highly variable V3 regions of the 16S rRNA gene were amplified by PCR from each biofilm DNA sample. One microliter of genomic DNA samples was amplified using the primers w49 (5'-ACGGTCCAGACTCCTACGGG-3', *Escherichia coli* position F330) and 5'-6FAM labelled w104 (5'-TTACCGCGCTGCTGCTGGCAC-3', *E. coli* position R533) [70] in accordance with previously described CE-SSCP amplification methods [71]. CE-SSCP electrophoreses were performed with ABI310 (Applied Biosystems). CE-SSCP profiles were analysed using

GeneScan software (Applied Biosystems) and the package 'Stat-Fingerprints' [72]. Pyrosequencing of the DNA samples using a 454 protocol was performed by Research and Testing Laboratory (Lubbock, USA).

2.5. Epifluorescence microscopy

EA-biofilm colonisation was imaged at the end of experiments by epifluorescence microscopy. The bioanodes were washed carefully with SMM (without carbon source) to remove all excess materials and chemicals, except for the attached biofilms. The biofilms were dyed according to Erable and Bergel [73], using 0.03% acridine orange (A6014, Sigma) for 10 min. Afterwards, the samples were rinsed with running demineralized water. The samples were analyzed with a Carl Zeiss Axiotech 100 microscope equipped for epifluorescence with an HBO 50/ac mercury light source and the Zeiss 09 filter (excitor HP450-490, reflector FT 10, barrier filter LP520). Images were acquired with a monochrome digital camera (Evolution VF) and treated with the Image-Pro Plus 5.0 software to recompose 3-D images.

3. Results and discussion

3.1. Functional halotolerant EA-bioanodes progressively deteriorate in natural marine media

An electrochemical reactor started up by polarizing a carbon felt anode (25 cm²) at 100 mV/SCE. After reaching a pseudo-steady-state behavior (~1 h), the reactor was inoculated with 10% v/v marine biofilm mix. An EA-biofilm progressively developed under natural seawater supplemented with 60 mM sodium acetate (NM₆₀). Every time the current dropped down almost entirely, the full exhausted medium was drained and replaced by an equivalent volume of fresh NM₆₀. This successive transfer was performed three times (Fig. 1). In the primary adaptation, current density (j) intensified up to about 6.8 A m⁻² during the first 10 days, followed by a progressive decay within the subsequent 8 days. After this drop in current density, the bioanode was transferred to fresh NM₆₀. Right after, a less significant current generation peak was attained (up to ~1.2 A m⁻²), with a total duration of nine days.

Concisely, successive transfers of halotolerant bioanodes into fresh natural media supplemented with a suitable carbon source resulted in progressive loss of the generated current. This behaviour was reproducible with 20 mM of sodium acetate as

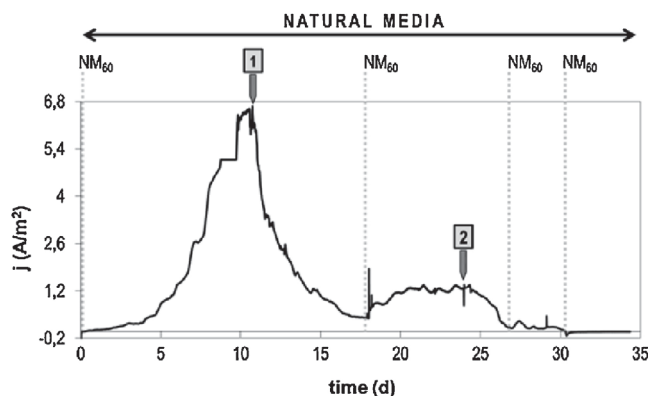


Fig. 1. Chronoamperometry of a marine biofilm developed over carbon tissue polarized at 100 mV/SCE. NM₆₀: Natural medium (seawater) supplemented with 60 mM sodium acetate. The bioanode was adapted to fresh NM₆₀; three successive transfers fresh NM₆₀ took place. Numbers pointing with arrows indicate moments when cyclic voltammograms were recorded.

carbon source, with an inferior performance for current generation. This behaviour is explained by depletion of vital nutrients other than the carbon source, for the EA-biofilm to sustain its activity.

3.2. The use of minimal media re-establishes current generation capacities of EA-biofilms that formerly deteriorated in natural media

An independent electrochemical reactor started up in the same conditions than formerly described for the system characterized in Fig. 1. The sole difference was that a third transfer to natural medium was avoided, in order to prevent total decay in current generation. Instead, the exhausted NM₆₀ was drained and replaced by a synthetic minimal media supplemented with 60 mM sodium acetate (namely SMM₆₀). In the primary adaptation and consecutive transfers to NM₆₀ (Fig. 2, days 0–26) the overall behaviour was consistent with the one observed during the total operation of the first reactor (Fig. 1): a first current peak with important current density was obtained, followed with subsequent peaks wherein current generation appears significantly deteriorated. However, right after transferring the bioanode into SMM₆₀, not only the current density doubled the magnitude of the precedent peak produced in NM₆₀ but also, after a second transfer to SMM₆₀, the current density attained equalled to the maximal level achieved with the initial NM₆₀ (Fig. 2, days 26–38). Furthermore, such level was maintained even after a third transfer to SMM₆₀ (day 38 onwards). Replicate experiments showed the same trend. To sum up, successive transfers of halotolerant bioanodes to a synthetic minimal media supplemented with a suitable carbon source, after progressive loss of current generation in natural media, resulted in recuperation of the original functionality. This behaviour was also reproducible with 20 mM of acetate, with an inferior maximal current density at the first peak (1–2 A m⁻²).

3.3. Preservation of electron transfer mechanisms is identified by cyclic voltammetry response

CVs were recorded at specific moments during the CA experiments presented in Fig. 1 and Fig. 2. Fig. 3 presents the derivatives of current density with respect to potential ($\Delta j/\Delta E$), for the oxidation turnoff as obtained in CV. With the purpose of improved

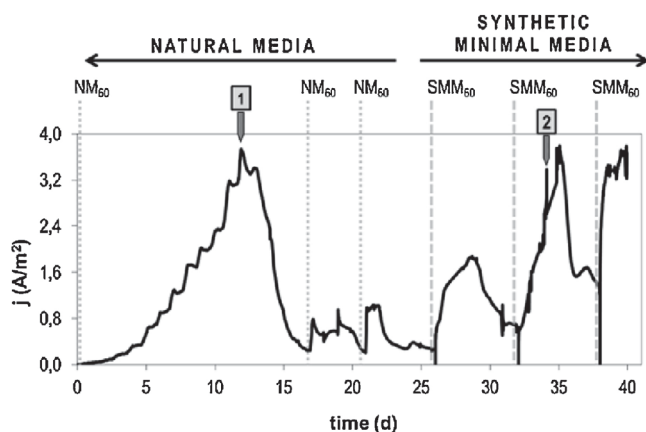


Fig. 2. Chronoamperometry of a marine biofilm developed over carbon tissue polarized at 100 mV/SCE. NM₆₀: Natural medium (seawater) supplemented with 60 mM sodium acetate. SMM₆₀: Synthetic minimal medium supplemented with 60 mM sodium acetate. The bioanode was first adapted to NM₆₀ with two successive transfers to fresh NM₆₀; subsequently it was repetitively transferred to SMM₆₀. Numbers pointing with arrows indicate moments when cyclic voltammograms were recorded.

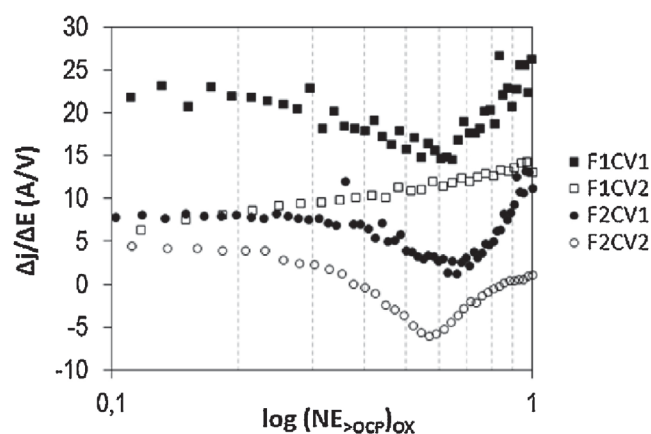


Fig. 3. Derivative of current density with respect to potential ($\Delta j/\Delta E$), for the oxidation turnoff as obtained in CV, beyond OCV. The CVs were performed at punctual moments, as shown on Fig. 1 (F1) and Fig. 2 (F2). CV1 or CV2 correspond to the points indicated with arrows in the corresponding Fig. 1 and Fig. 2.

visualization, only those values beyond open circuit voltage (OCV) are shown. For the same purpose, the potential is presented in logarithmic scale and normalized. First, it becomes clear that the mechanisms of electron transfer occurring for the oxidation of acetate during the first peaks of current generation obtained with NM₆₀, as presented in Fig. 1 and Fig. 2 respectively, are equivalent and just differ in intensity (Fig. 3, ■ and ●). When the EA-biofilm was consecutively transferred to NM₆₀ solely, not only current generation failed, but also it was evident that the electrochemical response of the system diverged (Fig. 3, □) when compared the one obtained in opposed circumstances where the biofilm was transferring current towards the carbon electrode (Fig. 3, ■, ●, and ○). In the case of the EA-biofilm adapted to SMM₆₀ (Fig. 2, day 26 onwards), it is appreciated that the electron transfer mechanisms (Fig. 3, ○) remain comparable to those formerly obtained with the primary colonizers in NM₆₀ (Fig. 3, ●), implying only a succession and not a modification of the functionality. This is, current density was impacted, but not the form of the kinetic response. To sum up, as observed from the transformation of the CV response (registered at different characteristic stages of the CA curves for different electrodes), it was confirmed that the halotolerant EA-bioanodes progressively exhausted their current generation functionality over the solid-state electrode when progressively exposed to natural marine media supplemented with a suitable carbon source, as opposed to the positive preservation and upsurge of such capacity when a total deterioration is prevented by prompt relocation in fresh minimal media supplemented with the same substrate.

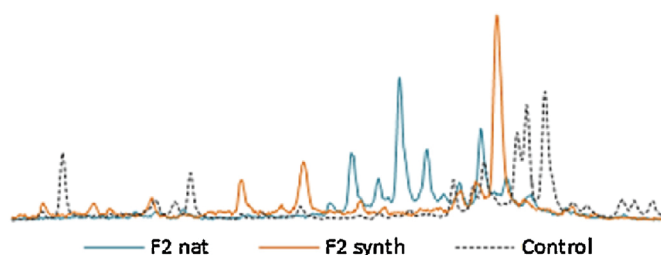


Fig. 4. CE-SSCP profiles showing the change of the microbial community structures. Microbial community exposed to natural medium (F2 nat) diverges to that observed for the inoculum (control) and the one from exposed to synthetic medium (F2 synth).

3.4. Preserved electrochemical functionality is not the outcome of fully preserved microbial diversity

CE-SSCP profiles presented in Fig. 4 correspond to the system whose electrochemical response is shown in Fig. 2 and Fig. 3 (F2). First, it can be appreciated that only a fraction from the original biofilm mix successfully colonized the carbon felt electrode and is correlated to the electrochemical activity observed. It can also be appreciated that although the electrochemical functionality is preserved and even recuperated at original levels (Fig. 2) after transfer of the bioanode to the synthetic minimal medium, this preservation and restoration is not associated to the prevalence of the same microbial community. Some of the predominant strains become extinct whereas others—poorly present at the primary stage with natural medium—boost after being transferred to the minimal medium.

Certainly, the presence/absence and concentration of certain nutrients such as nitrogen or certain oligoelements can favour the growth and performance of certain microbes. However, it is here believed that the induced stress given by the change in nutrient makes electron transfer towards the carbon electrode the predominant mechanism for survival. Given the conservation of the form of the CV response, as observed in Fig. 3, electron transfer could be attributed to a predominant electrochemical mechanism for this microbial population by either direct electron transfer or by entrapment of self-produced mediators in the extrapolymeric matrix.

3.5. Mild nutrient stress at the thin line between inactivation and intensification boosts the effect of minimal media

Bacterial stress can be defined as any deleterious physical, chemical or biological factor that induces modifications in the physiology of bacteria [74]. Adequate nutrient levels will promote metabolic progression at a maximum rate, which is characteristic for every microbe. Hence, variations in these levels represent an environmental stress factor. In fact, in their natural habitat, conditions that allow for maximal microbial metabolic rates are few and far between. Most marine biofilms are naturally adapted to live in a constant state of stress, due to nutrient scarcity and abundance (sometimes up to the point of toxicity) [75]. Even, fluctuating or seasonal conditions invariably place them in this “feast and starvation” existence, with the “hunger between meals” the more habitual state [76,77]. With this in mind, part of the present investigation consisted on simulating transition states between feast and famine, which entail exposure to an environment in which bacteria are limited for one or more classes of essential nutrients [78]; the *in-between hunger state*, would emphasize on bacterial scavenging for substrates to further stimulate nutrient acquisition. Of course, such strategy was combined with the standpoint of re-establishing the current generation functionality deteriorated in natural media, with the use of minimal media, as previously explained.

An independent electrochemical reactor started up in similar conditions than formerly described for the systems presented in Fig. 1 and Fig. 2, with a different type of carbon tissue as support material. The initial concentration of acetate was 20 mM (NM₂₀)—which is far from limiting.

After current decay of the first peak, the exhausted medium was removed (decanted) and consecutively replaced by fresh natural medium with increasing concentrations of acetate: NM₄₀, NM₆₀ and NM₈₀, respectively. Successive transfers to these fresh natural media negatively impacted on current generation, even when the concentration of acetate was progressively increased. This is consistent with the rationale that there are dominating important nutritional deficiencies in natural seawater, aside of the carbon

source needed by the EA-bacteria to proliferate. It should be noted that this behavior was observed independently of the type of carbon support used.

Acetate concentration was periodically followed for each peak of current production. Almost all acetate was depleted by the lapse of each transfer to fresh medium. Still, current density peaks did not intensify accordingly (Fig. 5). This may be explained by either of two possibilities: a) the planktonic counterpart was largely consuming the substrate through a fermentative route; b) the biofilm at the surface becomes more and more densely populated by non-electrochemically active microorganisms.

By day 21 (Fig. 5), current density was low and stable at about 0.25 A m⁻². At such moment, 50% of the volume of the medium was removed (containing about 10 mM of residual acetate), and replaced by fresh seawater. This step was executed in order to address if an immediate dilution of the planktonic population would improve electricity generation. Current density remained without progress for the following four days. Subsequently, the anode was re-transferred to fresh NM₂₀, since these were the conditions in which the EA-biofilm performed best during almost one month of experimentation. A slight improvement was observed with this action but, still, current density remained below 0.5 A m⁻² and did not resume to its initial highest.

It was anticipated from previous experiments that transferring the EA-bioelectrode to SMM would re-establish current generation. Still, in order to determine if greater nutritional variety (provided by yeast extract) would favorably impact current production as opposed to direct passage to non-supplemented SMM. The medium was decanted and fully replaced by SMM-Y₂₀, where Y refers to SMM supplemented with 0.4 g/L of yeast extract. As observed in Fig. 5 (days 38–49) such enrichment performed almost invariable with respect to preceding NM₂₀, confirming that nutrient-richer conditions do not specially favor current production for this system. Additionally, yeast extract could have introduced additional electron acceptors, thus deviating electron transfer from the anode to their electron-mediating structures.

After almost 49 days of operation, the bioanode was finally transferred to SMM₂₀. In the immediate couple of days current density started to visibly increase up to 0.7 A m⁻². What is more, in two consecutive transfers to SMM₂₀ the system not only re-established current generation up to about 2.8 A m⁻² and 3.4 A

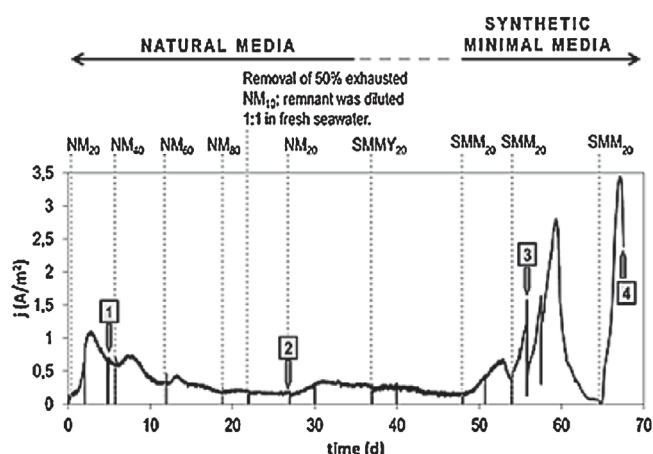


Fig. 5. CA of marine biofilm developed over carbon tissue. The bioanode was first adapted to NM₂₀ with successive additions of acetate at different concentrations (NM₄₀, NM₆₀ and NM₈₀). Subsequently, 50% of the exhausted media was removed (~NM₁₀ remained, presumably) and the remnant was diluted 1:1 with fresh seawater. Later on, the full exhausted media was replaced with again NM₂₀. Further the bioanode was transferred to SMM-Y₂₀. Since no current improvement was detected after these stress factors (~day 48), the bioanode was finally successively transferred to SMM₂₀, showing significant increase in current density.

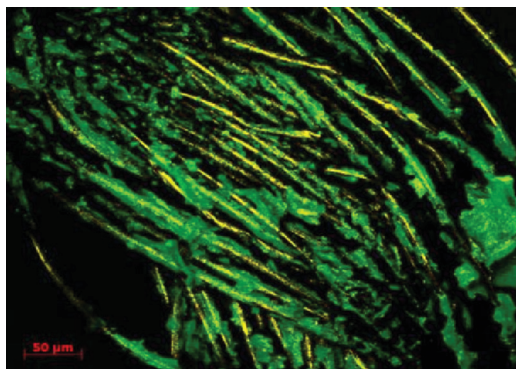


Fig. 6. 3-D Structure of an halotolerant anodic biofilm colonization over carbon tissue fibres, after 67 days of polarization at 100 mv/SCE, as shown in Fig. 4. Biofilm aggregation (green) is perceived around the carbon tissue fibers (yellow).

m^{-2} , respectively, but it surpassed by more than three times the current density initially obtained in NM_{20} . An independent replicate reactor showed a comparable trend.

An epifluorescence microscopy image is presented (Fig. 6), showing representative biofilm colonization over the carbon tissue fibers at day 67. Biofilm aggregation was found to be significant around the carbon-fibers, with an estimated thickness of about 20–30 μm , which is typical in bioelectrochemical systems [79]. Over the typically used carbon felt, this type of marine biofilms have shown 80–90 μm thickness [80] in natural media.

3.6. Nutritional stress factors by conditions fluctuating from natural to enriched to synthetic minimal medium preserve and boost current generation functionality

CE-SSCP profiles presented in Fig. 7 correspond to the system presented in Fig. 5, at two different stages (1, Stress nat, and 4, Stress synth). In general, the same behaviour shown in Fig. 4 was observed. Only a fraction from the original biofilm mix successfully colonized the carbon felt electrode. Similarly, the electrochemical functionality was preserved and recuperated, but in this case the additional stress due to fluctuating from a natural medium, to excess and further to deprived minimal conditions, significantly improved current density generation. At least 7 peaks are preserved from the initial population developed in natural medium. Such peaks were also present in the EA-biofilm subject of Fig. 4 at a lower extent. In both cases such peaks were enhanced when the bioelectrode was transferred to minimal media, as compared to their significance in natural media. It is possible that despite of the overall changing microbial community a set of microorganisms which are truly electrochemically active over solid-state carbon electrodes are much favoured by the nutrient

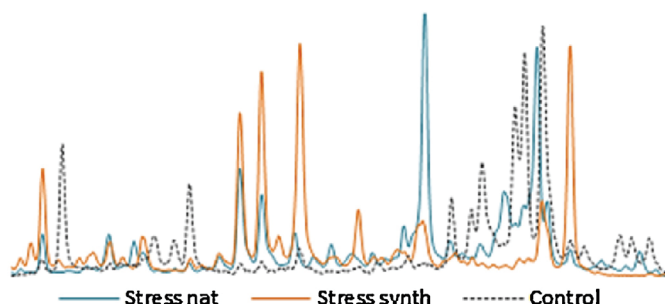


Fig. 7. CE-SSCP profiles showing the changes on the microbial community structure. The microbial communities exposed to natural media (Stress nat) diverge from those observed for the original inoculum source (control) and the one exposed to synthetic media after exposure to different stress factors (Stress synth).

minimal condition and even further when such is provided after nutrient-fluctuating stress conditions.

3.7. Implications of these results

The results here disclosed reveal a simple, yet innovative, strategy to boost current generation in independent microbial anodes for electrochemical systems. Development of microbial biofilms for effective electro-bioprocesses must be robust and capable to work with minimal requirements; besides, they should display high functionality and reproducibility when operating in normal capacity and be able to endure periodic stress conditions. Only such types of biofilms could become not only industrialized but polyvalent for different types of BES and expand their use to new areas and markets. The EA-biofilms here studied presented these characteristics when subject to the developed procedure. However, this first long-term research of halotolerant microbial anodes could be still improved, playing with seasonal variations on the original inoculum or shifting batch to continuous mode operation. Improving the overall current generating capacity of these robust and versatile bioanodes is critical. Future work may consider continuous operation and identification of the parameters that will improve current densities, such as nature and concentration of the carbon source, electrode material, and start-up electrode potential, osmotic and hydrodynamic conditions, among others. Further elucidation of mechanistic implications of how EA-biofilms cope with nutritional stress would also advance this scientific field.

Finally, it is relevant to state that replicate experiments referred in this manuscript showed the same trend than the independent experiments here shown, even when different types of carbon support materials and different substrate concentrations were employed. Variability in the magnitudes of current density was encountered. Still, such variability did not have an impact in the main findings:

1. EA-marine anodes progressively deteriorate in natural marine media supplemented with acetate, not in terms of biomass growth but in their functionality to generate sustained current.
2. The use of a minimal synthetic feed supplemented with acetate, re-establishes current generation capacities of the marine EA-marine anodes that formerly deteriorated in natural media.
3. The overall electron transfer mechanisms for oxidation reactions are preserved when passing from natural to a suitable synthetic media, as evidenced from the CV responses.
4. Progressively-induced mild nutrient stress while the EA-biofilm deteriorates in natural media not only restores current generation when transferring to minimal media, but also boosts its influence leading to unprecedented current densities for an individual bioanode.

4. Conclusions

Electrochemically active biofilms were developed in synthetic minimal media showing effective performance in terms of current density (e.g. up to 3.8 A m^{-2} to 6.8 A m^{-2}), when compared to their deteriorated performance in natural media (e.g. wherein the current density dropped below 0.1 A m^{-2}). Even, when nutrient stress was induced, the use of synthetic minimal media not only restored but boosted current density.

The use of minimal culture media should carefully be considered for industrialized METs, especially in what concerns microbial electrolysis for hydrogen generation and microbial electrosynthesis for product organics, as these would permit more straight forward downstream processing.

Change from natural to minimal media has an important impact on the selection and adaptation of microbial communities; however, robust bioanodes containing some preserved species were obtained in synthetic minimal media and showed reproducible electrochemical functionality.

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