Forming microbial anodes under delayed polarisation modifies the electron transfer network and decreases the polarisation time required.

Diana Pocaznoi, Benjamin Erable, Luc Etcheverry, Marie-Line Délia-Dupuy, Alain Bergel

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Diana Pocaznoi *, Benjamin Erable, Luc Etcheverry, Marie-Line Delia, Alain Bergel

Laboratoire de Génie Chimique CNRS-Université de Toulouse (INPT), 4 allée Emile Monso BP 84234, 31234 Toulouse, France

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A B S T R A C T

Microbial anodes were formed from compost leachate on carbon cloth electrodes. The biofilms formed at the surface of electrodes kept at open circuit contained microorganisms that switched their metabolism towards electrode respiration in response to a few minutes of polarisation. When polarisation at −0.2 V/SCE (+0.04 V/SHE) was applied to a pre-established biofilm formed at open circuit (delayed polarisation), the bacteria developed an extracellular electron transport network that showed multiple redox systems, reaching 9.4 A/m^2 after only 3–9 days of polarisation. In contrast, when polarisation was applied from the beginning, bacteria developed a well-tuned extracellular electron transfer network concomitantly with their growth, but 36 days of polarisation were required to get current of the same order (6–8 A/m^2). The difference in performance was attributed to the thinner, more heterogeneous structure of the biofilms obtained by delayed polarisation compared to the thick uniform structure obtained by full polarisation.

1. Introduction

Electro-active (EA) biofilms have been widely exploited to design microbial fuel cells (MFCs) (Lefebvre et al., 2011; Logan, 2010) microbial electrolysis cells (Geelhoed et al., 2010; Logan et al., 2008) and other related technologies. Numerous studies have identified EA bacteria (Logan, 2009) and deciphered the different electron transfer pathways that can be used inside microbial biofilms (Rabaey et al., 2007; Reguera et al., 2005; Schaetzle et al., 2008). In contrast, the mechanisms of formation, structuring and ageing of the EA biofilms have rarely been investigated. Wang et al. (2009) have observed that the start-up time of an MFC was significantly decreased when a constant potential was applied to the anode, in comparison with the same MFC implemented without applied potential. They postulated that the applied potential increased the positive charge on the anode surface and thus favoured the primary adhesion of negatively charged bacteria. The adhesion of EA species on anodes might consequently depend on the surface charge of the electrode as it has sometimes been suggested (Busalmen et al., 2008; Cheng and Logan, 2007). In contrast, Aelterman et al. (2008) did not observe significant difference in start-up time with applied potentials in the range from −0.4 to 0 V vs. Ag/AgCl. Actually Wang et al. (2009) have inoculated their MFCs with domestic wastewater, while Aelterman et al. (2008) have taken their inoculum from an operating MFC, which means that the microbial community was already adapted to generating electricity. Comparing the two studies suggests that the time necessary for wild microbial communities to adapt to electrochemical conditions depends on the applied potential, while the time necessary for already-adapted bacteria to form EA biofilms does not. When using wild communities as inoculum, the adaptation phase necessary for the cells to develop their EA capacity may consequently be an essential parameter in controlling the formation of EA biofilms, rather than electrostatic interactions between bacteria and electrode surface. The occurrence of an initial phase during which the cells must optimize attachment or the electron transfer chain to the surface has also been observed with pure cultures of *Geobacter sulfurreducens* (Marsili et al., 2010). Nevertheless, little is known so far about the way a clean electrode surface catches microbial species from a natural environment and how they shift from the conventional respiration mechanism to an anode respiring mechanism.

The purpose of this work was to give some insights into EA biofilm construction with the practical target of improving the performance of microbial anodes. Garden compost was used as the source of the inoculum. Soils are a very rich source of microorganisms (Liu et al., 2006; Torsvik et al., 1996) and garden compost has proved its excellent capacity to form EA biofilms. Microbial anodes can be formed by simply embedding polarised electrodes in a soil (Parot et al., 2008), but it is then difficult to use the resulting anodes out of their initial medium. A new procedure has been proposed recently, which consists of producing a leachate by percolating the garden compost with an ionic solution and then using the

* Corresponding author. Tel.: +33 5 34 32 36 27; fax: +33 5 34 32 37 00.
E-mail address: diana.pocaznoi@ensiacet.fr (D. Pocaznoi).

leachate obtained as the inoculum. This procedure has given promising results for the treatment of dairy wastes (Cercado-Quezada et al., 2011, 2010a). The same procedure has led to current density so high as 66 A/m^2 for acetate oxidation when the biofilms were formed around ultra-microelectrodes (Pocaznoi et al., 2012). Obviously this result has been reached with particular laboratory electrodes, but it demonstrates the promising potential of the inoculum source.

The present study used compost leachate as inoculum and acetate as substrate. Comparing biofilms formed at open circuit and biofilms formed under constant polarisation showed very different structures and allowed the phases of biofilm formation and of development of the extracellular electron transfer network to be distinguished. These observations were exploited to define an optimal procedure for the formation of microbial anodes.

2. Methods

2.1. Soil biofilm

One litre of Garden compost (Cultura, Lombricompost) was mixed with a 60 mM KCl solution and left for 24 h under stirring at room temperature. The mix was then centrifuged and the resulting leachate, supplemented with 10 mM acetate, was used for the formation of EA biofilms.

2.2. Electrochemical instrumentation and set-up

Electrochemical experiments were carried out in closed vessels that contained 150 mL leachate. Carbon cloth (supplied by PaxiTech SAS, France) of 2 cm^2 projected surface area was used for the working electrodes and a platinum grid for the auxiliary electrode. All potentials were controlled via a conventional 3-electrode set-up vs. a saturated calomel reference electrode (SCE, Radiometer, +0.241 V vs. SHE) by means of a VMP potentiostat (Bio-logic SA). Polarisations were performed at −0.2 V vs. SCE because this potential was around the most negative value that could provide the maximum current density (Cercado-Quezada et al., 2010b). Cyclic voltammetries were performed at 1 mV s^{-1}. Electrochemical experiments were carried out at room temperature, around 22 °C.

2.3. Microscopy and image analysis

For scanning electron microscopy, the microbial structures were stabilised on the electrode surfaces by fixation in phosphate buffer (400 mM, pH 7.0) with 4% glutaraldehyde. The electrodes were then rinsed in phosphate buffer with saccharose (0.4 M), treated with 2% osmium tetroxide in phosphate buffer and saccharose for 1 h, dehydrated in an ascending series of acetone solutions (50%, 70%, 100%), then in acetone and hexamethyldisilazane (50:50) and, finally, in 100% hexamethyldisilazane. Anodes were examined with an LEO 435 VP scanning electron microscope.

3. Results and discussion

3.1. Biofilm formed at open circuit

Five 2 cm^2 carbon cloth electrodes were left at room temperature in separate bioelectrochemical reactors containing 150 mL of the same compost leachate supplemented with 10 mM acetate, for 0 (control), 8, 12, 15 and 19 days. No polarisation was applied and the open circuit potential (OCP) was recorded as a function of time (Fig. 1A). The OCP exhibited similar evolution for each electrode, with initial values around +0.05 V vs. SCE and a fast decrease during a 4-day period, followed by a stabilisation at close to −0.5 V vs. SCE at day 5.

The OCP values are the result of the delicate balance between electron exchanges that are spontaneously established between the electrodes and the species from the bulk environment. For instance, cathodic currents (electrons lost by the material) may be due to the natural slow reduction of dissolved oxygen on the electrode surface, and anodic currents (electrons gained by the material) may result from the slow oxidation of the electrode surface itself. With graphite and carbon electrodes, the presence of functional groups like phenol, carbonyl, carboxyl and quinine on the surface (Cabaniss et al., 1985; Nagaoka and Yoshino, 1986) makes the anodic process complex. Moreover, in a solution such as compost leachate, which contains a large diversity of dissolved organic compounds, anodic currents can also be due to the slow spontaneous oxidation of some of them. For instance, OCP recording is commonly exploited to measure the redox potential of solutions. In this case, a platinum electrode is used because no significant oxides form on its surface.

From a general point of view, OCP decrease is due to vanishing of the reduction current and/or to an increasing of the anodic current. For instance, in redox potential measurements with a platinum electrode, it is generally postulated that OCP decrease is mainly due to vanishing of the cathodic current because of oxygen depletion. Here OCP decrease can be due to both the oxygen depletion in the closed electrochemical reactor and the increase of the anodic current resulting from the catalysis of acetate oxidation. Actually, it should be borne in mind that OCP values are controlled by the very low exchange currents due to spontaneous reactions only.

In this framework, the adhesion of only a few EA bacteria is sufficient to catalyse low acetate oxidation and to cause OCP decreases observed here. To check this assumption, two experiments were performed with, in each reactor, one electrode dipping in the leachate and another hanging in the gas free space. In both experiments, the OCP of the immersed electrodes followed the same evolution as previously recorded, with an initial OCP around +0.1 V vs. SCE which decreased to −0.65 V vs. SCE after 20 days of immersion. At day 20, the hung clean electrodes were plunged into the solutions. The OCPs of the clean electrodes were in the range of −0.1 to −0.2 V vs. SCE in both experiments, i.e. around 0.2 mV less than the initial OCP value. The difference between the initial OCP value (+0.1 V vs. SCE) and OCP measured at day 20 with the clean electrode can only be attributed to changes in the solution composition, and most probably to the consumption of oxygen by the aerobic microorganisms during the first few days of the experiment. The final stable OCP values being around −0.65 V vs. SCE, it can be concluded that oxygen depletion was responsible for around one third of the whole OCP decrease, while the catalysis of the oxidation reaction supported around two thirds. These experiments confirmed that a major part of OCP decrease was due to the primary settlement of EA microbial cells that started to catalyse acetate oxidation.

Going back to the five-electrode experiment, each electrode was polarised at −0.2 V vs. SCE for 3 h at the end of its open circuit phase (Fig. 1B). The control electrode that was polarised immediately upon immersion in the compost leachate did not produce any current during the 3 h of polarisation. In contrast, every other electrode gave an oxidation current density of about 50 mA/m^2 after only 30 min of polarisation. This time was too short for the current increase to be attributed to the growth of EA microbial species. It indicated an activation phase of the EA cells that were already present on the anode surface and switched their metabolism towards an electrode-respiring mechanism in response to the polarisation.

In conclusion, the microbial communities that adhered to the surface of non-polarised electrodes contained some microbial

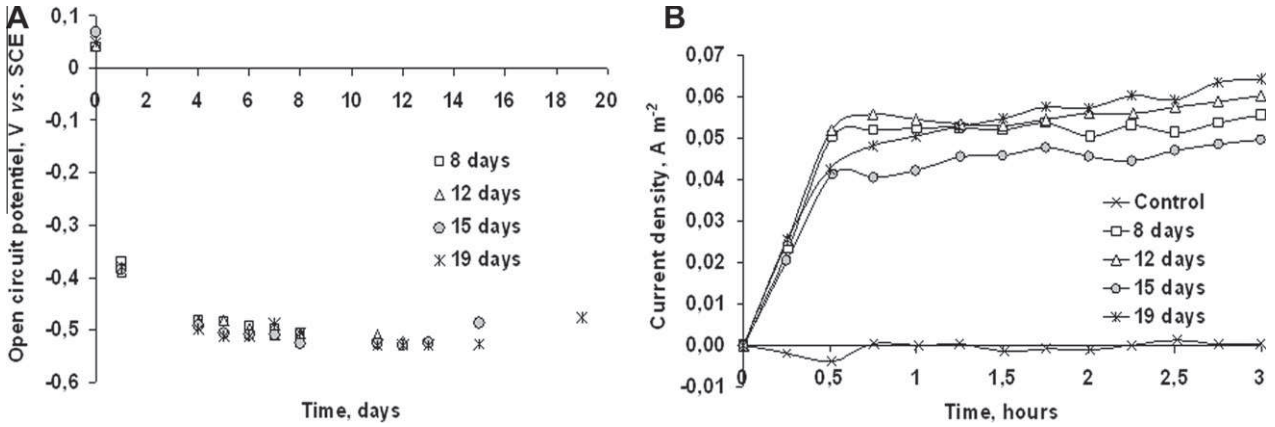

Fig. 1. Biofilms formed at open circuit. (A) Evolution of open circuit potential (OCP) of carbon cloth electrodes immersed in compost leachate for 8, 12, 15, and 19 days. (B) Current densities obtained during 3 h polarisation at −0.2 V vs. SCE on the 8-, 12-, 15- and 19-day old biofilms.

species with EA ability, which contributed to OCP decrease. These EA species activated their capability to use the electrode as final electron acceptor after a few tens of minutes of polarisation.

3.2. Biofilm formation at open circuit followed by short-term (3-day) polarisation

Six new reactors (150 mL compost leachate, 10 mM acetate) were started with six 2-cm^2 carbon cloth electrodes kept at open circuit for different times: 1, 5, 9, 12, 15 and 19 days. At the end of the open circuit time, a new 10 mM acetate addition was made and each electrode was polarised for 3 days at −0.2 V vs. SCE. The final biofilm ages were respectively 4, 8, 12, 15, 18 and 22 days. The current densities measured at the end of the 3 days of polarisation increased from 0.2 to 2.7 A/m^2 with the biofilm ages (Fig. 2A). This experiment was reproduced with a leachate formed from a different brand of compost. After 3 days of polarisation the biofilms with final ages 4, 7, 10 and 12 days gave 0.1, 0.4, 0.9 and 2.2 A/m^2, respectively. This second compost led more quickly to efficient biofilms, but the general behaviour was the same: the biofilms obtained after 3 days of polarisation always showed more efficient EA capabilities when the preliminary open-circuit time was longer.

Cyclic voltammetry (CV) was performed at the end of the 3-day polarisation (Fig. 2B). The current densities measured at −0.2 V vs. SCE on the CV curves were identical to the values recorded at the end of the polarisation, this showed that the CV scan rate was low enough to represent the stationary behaviour of the microbial electrodes.

CV curves detected a variety of different electrochemical characteristics. Some electrodes exhibited a conventional sigmoid shape (4- and 18-day-old biofilms), while others showed one or several peaks (12- and 22-day biofilms). Peaks observed with the 12-day-old biofilm revealed the presence of different electron transfer pathways in comparison to the other biofilms. The variety of different CV shapes revealed differences of the biofilms in terms of EA capabilities. Compost is a microbiologically-rich medium and the spontaneous formation of biofilms without any particular selection pressure during the open circuit phase logically led to different biofilms in terms of EA capabilities. Moreover, after 3 days of polarisation, biofilms were at the crossroads of their development, tipping over from an open-circuit way of development to polarisation-supported growth. Actually, EA and non-EA species that had spontaneously colonised the electrode surface during the open-circuit phase were still present in the biofilm, while the 3-day polarisation started to exert a selection pressure. CVs recorded at this crucial period consequently detected diverse EA capabilities. Nevertheless, whatever the differences in the intrinsic characteristics of each biofilm, it was observed that a longer preliminary open-circuit phase favoured the emergence of EA properties. The older biofilms (18 and 22 days old) produced significant current density (1 and 2.8 A/m^2, respectively) after only 3 days of polarisation. The 22-day-old biofilm exhibited a characteristic shape that has been reported in a recent

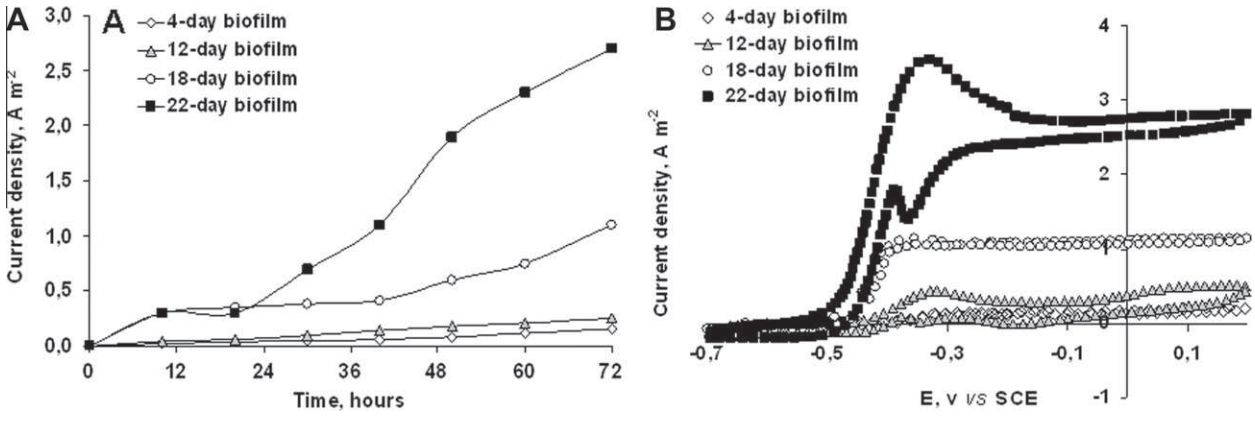

Fig. 2. Biofilms formed at open circuit followed by 3 days of polarisation at −0.2 V vs. SCE. (A) Current densities obtained during 3 days of polarisation on biofilms formed at open circuit. The final ages 4, 12, 15, 18 and 22 days given on the figure include the 3 days of polarisation. For clarity, the current provided by the 8-day old biofilm located between 4- and 12-day old biofilms have not been plotted. (B) Cyclic voltammograms (1 mV s^{-1}) recorded at the end of the 3-day polarisation.

theoretical study (Strycharz et al., 2011). This study described the electron transfer (ET) pathway from acetate to the electrode in 5 steps:

(i) mass transport of acetate and products,
(ii) reduction of the microbial cells by acetate,
(iii) reduction of (an) electron transport mediator(s) by the reduced microbial cells,
(iv) electron transport by mediator diffusion (for diffusing mediators) or by a series of sequential redox reactions between adjacent molecules of mediators (for bound mediators),
(v) oxidation of the mediator(s) at the electrode surface.

Theoretical CV curves were derived assuming for each that one of the five different steps was rate-limiting. Some numerical CV curves showed the same shape as that observed here with the 22-day biofilm, with an oxidation peak superimposed on the conventional sigmoid curve. Such curves were obtained only when the ET rate was assumed to be limited by step (iii) i.e. by ET from the microbial cell to the mediator. The numerical model never led to a similar peaked curve when the other steps were assumed to be rate-limiting. This hypothesis makes sense here. The biofilms that were formed without polarisation during the first 19 days did not develop an extracellular ET network. After 3 days of polarisation, the cells that had potential EA capability had shifted their metabolism to the anode-respiring pathway, they had probably started to multiply, but they had not yet produced an efficient extracellular ET network. They needed more time to develop an efficient ET network through the initial biofilm that had formed during the 19 days at open circuit. The ET rate can consequently be limited by step iii, i.e. by electron transfer from the microbial cells to the still poorly developed ET network. The younger biofilms did not show this limiting step because the initial open-circuit biofilm was less developed. Comparing the CV curves obtained here with the theoretical model led to the conclusion that applying the polarisation on a pre-established biofilm formed at open circuit induced two successive development phases: the EA cells present in the biofilm firstly turned their metabolism to the anode-respiring pathway and then they developed the extracellular ET network in a second phase.

At the end of the 3-day polarisation, the biofilms (final ages 4, 8, 12, 15 and 18 days) were imaged by scanning electron microscopy (SEM). For each microbial electrode, the surface of the weft of the woven carbon cloth was always visible at low magnification (Fig. E1A to E1E). The surfaces exhibited poor colonisation by scattered bacterial colonies. The lower magnification showed visible coating only with the 18-day old biofilm (Fig. E1E), which was consistent with the currents obtained during 3-day polarisation. Significant currents started to be obtained with the 18-day biofilm (final age), while no significant current was provided by the younger biofilms (Fig. 2A). Higher magnification showed that the microbial colonies were locally more important on biofilms aged of more than 8 days. Cells with flagella were observed in 12- and 15-day old biofilms (Fig. E1C-D). Planktonic cells use flagella to move in solution, to approach solid surfaces and to fix onto them but the flagella disappear rapidly when the cells are embedded into a biofilm. The presence of flagella observed here revealed an early stage of biofilm formation (Monroe, 2007). As discussed above, these biofilms were at the crossroads of their development. The 3 days of polarisation had not yet erased the differences in colonisation that resulted from the different open circuit durations, and the EA bacteria that were able to multiply during the 3 days of polarisation were not yet predominant enough to mask the biofilm development by the uptake of planktonic cells with flagella.

3.3. "Delayed polarisation" vs. conventional "full polarisation"

The 22-day-old biofilm, which supplied 2.7 A/m^2 after the 3 days' polarisation, continued to be polarised for a total of 20 days. When the current dropped off because of acetate depletion, 10 mM acetate was added and the current rapidly increased again. A current density of 9.4 A/m^2 was reached at day 28, i.e. after only 9 days of polarisation (Fig. 3A, biofilm 1). The 3rd acetate addition confirmed an identical maximum current density. A replicate with a first period of 20 days at open circuit gave a maximum current density of 9.4 A/m^2 from the first current peak, i.e. after only 3 days of polarisation (Fig. 3A, biofilm 2). These biofilms were said to be obtained by "delayed polarisation". This new strategy was attempted here with the objective of producing high performance microbial anodes. It consisted of keeping the working electrode at open circuit for several days (here 19–20 days) before applying polarisation at −0.2 V vs. SCE.

In parallel, microbial anodes were developed following the conventional "full polarisation" procedure, which consists of polarising the electrode at −0.2 V vs. SCE as soon as it is immersed in the compost leachate. The current, which was initially nil, started to increase after several days due to the formation of the EA biofilm. The successive additions of 10 mM acetate improved the current (Fig. 3B, biofilm 3). A maximum current density of 6.0 A/m^2 was observed at the 4th acetate addition, i.e. after 36 days of polarisation. This experiment was duplicated (Fig. 3B, biofilm 4) and gave 8 A/m^2 after 35 days of polarisation and four acetate additions.

A preliminary conclusion is that delayed polarisation allowed maximum current density up to 9.4 A/m^2 to be reached after only 3 days of polarisation, while conventional full polarisation required 35 days of continuous polarisation to obtain 6–8 A/m^2. Waiting several days at open circuit before applying the potential had a positive effect on the performance of the microbial anode. The increase in current density was not high, but the decrease in the required polarisation duration was drastic.

3.4. Catalytic (i.e. in the presence of acetate) and non-turnover (i.e. in acetate depleted conditions) cyclic voltammetry of biofilms prepared by delayed and full polarisation

During the experiments described in Fig. 3A and B the polarisation was interrupted from time to time to record cyclic voltammetry (CV) curves at different steps of biofilm development. Catalytic CVs were plotted when the electrode provided maximum current density, **days 22 and 35** for the biofilm obtained by delayed polarisation (Fig. 4A) and **days 12, 30 and 36** for the biofilm obtained by full polarisation (Fig. 4B). Once again, the current densities measured at −0.2 V vs. SCE on the CV curves were identical to the values recorded during polarisation, which means that CV gave a correct representation of the stationary behaviour of the electrodes. The anodes showed excellent electrochemical characteristics, with low OCP around −0.50 V vs. SCE and high current densities. The higher current densities provided by the microbial anodes formed by delayed polarisation were confirmed.

All CVs recorded on the full-polarisation biofilm exhibited a conventional sigmoid shape, similar to those already reported in the literature, for instance for *Geobacter sulfurreducens* biofilms (Fricke et al., 2008) or for microbial anodes obtained from domestic wastewater (Liu et al., 2010). These biofilms did not show the peak system characteristic of the step iii) rate-limiting effect. CVs did consequently not detect a rate-limitation by electron transfer from the reduced cells to the ET mediator(s). Under constant polarisation the EA cells grew by using the electrode as final electron acceptor and they consequently developed concomitantly with their growth an extracellular ET network that perfectly matches

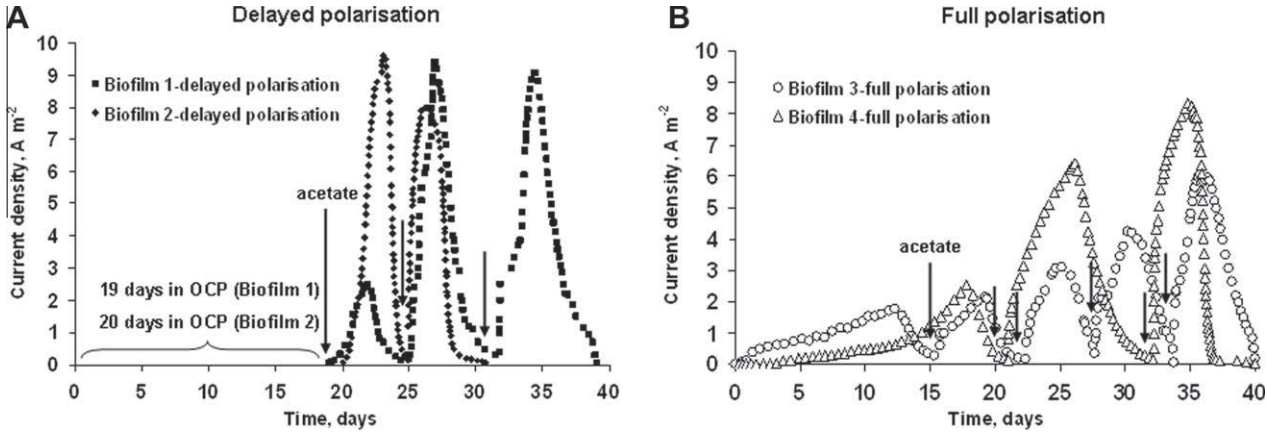

Fig. 3. Current densities under polarisation at −0.2 V *vs.* SCE. (A) Delayed polarisation after 19 or 20 days let at open circuit, (B) Conventional full polarisation.

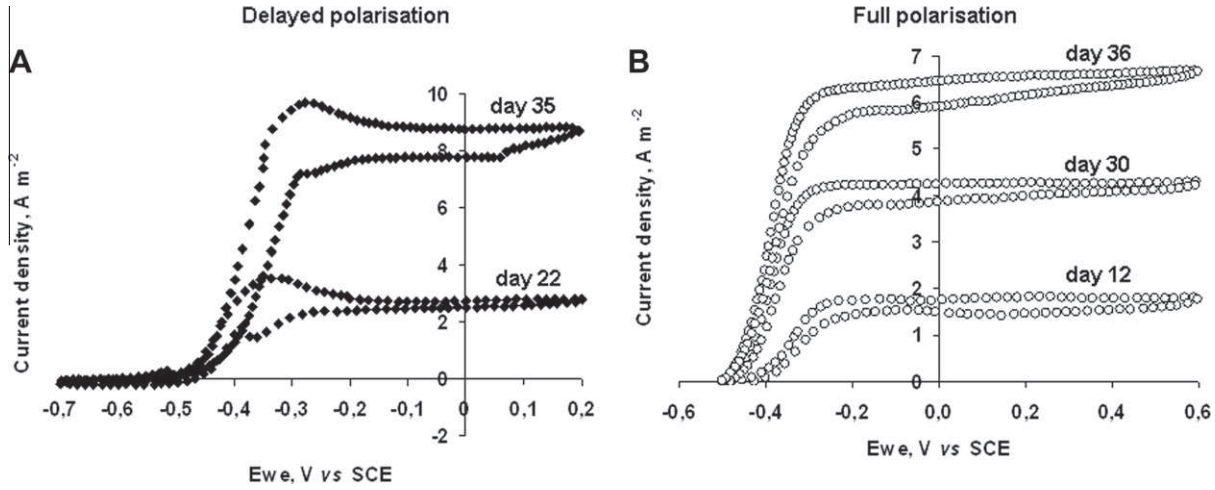

Fig. 4. Cyclic voltammograms (1 mV s^{-1}) recorded at different days around maximal current production on: (A) biofilm formed by delayed polarisation (Fig. 3A, Biofilm 1), (B) biofilm formed by full polarisation (Fig. 3B, Biofilm 3). Days of CV recording are indicated on the figures.

their needs. In contrast, when applying polarisation to a pre-established biofilm formed by 19 days at open circuit, CVs showed a superimposed oxidation peak that indicated a step (iii) rate limitation. The superimposed oxidation peak was observed after 3 days of polarisation (Fig. 4A, day 22) and was less marked but still present after 13 more days of polarisation (Fig. 4A, day 35). The rate-limiting effect of electron transfer from the microbial cells to the ET network slightly diminished during polarisation, but it durably marked microbial anodes obtained by the delayed polarisation procedure.

Non-turnover CVs were recorded in acetate-depleted conditions to gain information about the redox species contained in the biofilms. Actually a small amount of acetate was still present in solution and weak catalytic currents were always visible on the CV curves. Nevertheless, the small currents due to the catalytic oxidation of acetate did no longer mask the currents due to the single turnover oxidation and reduction of the redox species of the biofilm. During the delayed polarisation procedure, the non-turnover CVs recorded just at the end of the open circuit phase (Fig. 5A – day 19) showed the absence of any significant redox couple. In contrast, after 20 days of polarisation the biofilm exhibited multiple redox systems (Fig. 5A – day 39): two clear reduction peaks and three barely defined oxidation peaks were detected, which gave redox systems with midpoint potentials close to −0.24 and −0.15 V vs. SHE. Similar patterns have already been reported in the literature for non-turnover CVs, even pure cultures have revealed complex behaviour with multiple redox systems (Fricke et al., 2008). For instance, CV at 1 mV/s on *Geobacter sulfurreducens* biofilm formed on graphite electrode has shown four redox systems with potential midpoints at −0.32, −0.18, −0.10 and +0.25 V vs. SHE (Strycharz et al., 2011). It would not make sense to compare CVs performed in different solutions, with different electrode materials and different microbial cultures, but the potential range in which the redox systems were observed here remains close to the value reported for *Geobacter sulfurreducens*. Clearly, the non-turnover CVs indicated that the biofilm gained rich redox content during the polarisation time. The 20-day polarisation enriched the biofilm in redox species remarkably. Nevertheless, the catalytic CVs performed just before acetate depletion (Fig. 4A, day 35) showed a remaining rate-limitation by electron transfer from the cells to the ET network. It must be concluded that the ET network developed during delayed polarisation was composed of multiple redox systems that did not perfectly match the requirements of the cells.

For comparison, biofilm 3 developed under 40 days of full polarisation showed only two redox systems, the midpoint potential of one being around −0.13 V vs. SHE (Fig. 5B) and the currents were very much lower than those recorded with the delayed-polarisation biofilm of the same final age (19 days open circuit +20 days

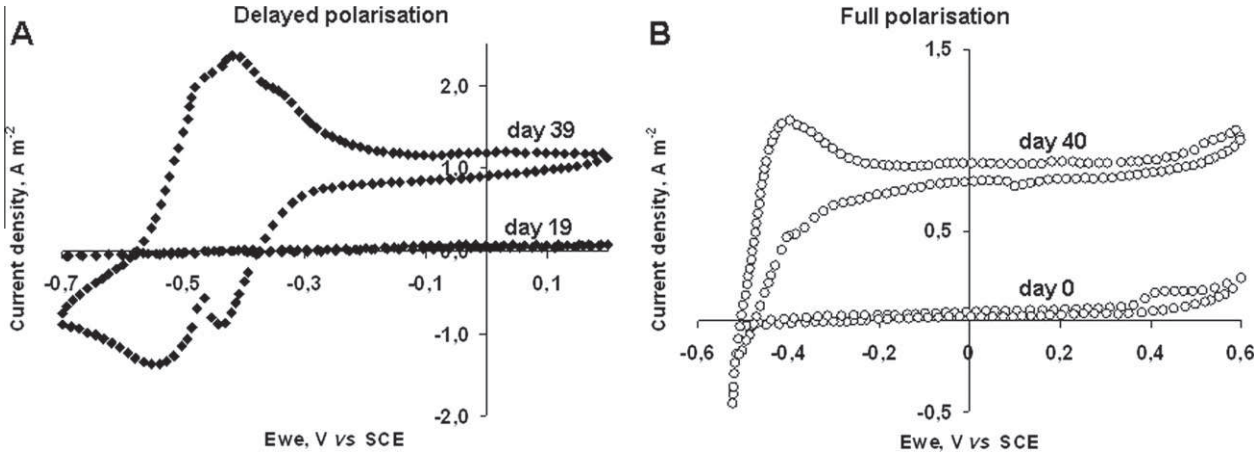

Fig. 5. Cyclic voltammograms (1 mV s^{-1}) recorded in acetate depleted conditions on (A) biofilm formed by delayed polarisation (Fig. 3A, Biofilm 1), (B) biofilm formed by full polarisation (Fig. 3B, Biofilm 3). Days of CV recording are indicated on the figures.

polarisation, Fig. 5A). The charge exchanged, which is proportional to the concentration of the redox species involved in the redox reaction, was drastically lower. The biofilm formed by 40 full days of polarisation showed, at the same final age, less intense electron transfer capabilities than the biofilm formed by delayed polarisation, but no limiting effect due to step (iii). This difference shows that the ET network was less rich for the biofilms formed under full polarisation but better suited to the EA cells that formed the biofilm.

Biofilms formed by delayed polarisation provided high current densities (up to 9.4 A/m^2) using an abundant ET network that was not optimally tuned to the microbial cells making up the biofilm, while the biofilm formed by the conventional full polarisation gave slightly lower current density (6–8 A/m^2) by using a less rich ET network well-suited to the microbial cells. Polarisation applied to a pre-established biofilm favours the development of different microbial species that develop different ET strategies. Nevertheless, the greater diversity does not ensure an optimal link between microbial cells and mediators. Applying the potential from the beginning of biofilm formation results in better tuned EA biofilms, but which provide lower current density.

3.5. SEM imaging of biofilms prepared by delayed and full polarisation

At the end of the 19 days' open circuit and 20 days' polarisation, the biofilm prepared under delayed polarisation showed a heterogeneous structure with clumps of bacteria but also many isolated single cells (Fig. E2A in Electronic Annex). The carbon fibres that made up the cloth electrode were still easily visible. Contrarily, the biofilm prepared under 40 days' full polarisation fully masked the fibre structure of the electrode, which was no longer visible (Fig. E2B in Electronic Annex). The biofilm was thick, uniform and covered the whole electrode surface area. Higher magnification confirmed its compactness.

SEM images showed that full polarisation drove the formation of a more compact and uniform biofilm than delayed polarisation. The biofilms obtained by delayed polarisation presented a globally thinner structure with heterogeneous coating. It can be thought that during the initial 19 days at open circuit non-EA bacteria settled the electrode surface and subsequently these bacteria hindered the access of EA bacteria to the electrode surface. The presence of non-EA bacteria resulted in a less dense and compact biofilm structure that gave higher current density. A mixed biofilm composted of EA and non-EA bacteria would thus be more efficient because of a more appropriate morphology.

3.6. Final scenarios

These differences in biofilm structures between delayed and full polarisation complete the assumptions derived from the electrochemical analyses. Polarising from the beginning favoured the growth of sessile EA cells that were able to use the electrode as final electron acceptor. They grew fast, formed a compact biofilm with an ET network that closely matched their need. The compact structure that was evidenced by SEM suggested that mass transfer of substrate and products inside the biofilm could become a rate-limiting step for these electrodes. For instance, it is known that slow extraction of protons produced by substrate oxidation can limit the current provided by microbial anodes (Torres et al., 2008). The theoretical model discussed above (Strycharz et al., 2011) has demonstrated that, in case of mass transfer rate-limitation, CV curves exhibit a purely sigmoid shape. The assumption of mass transfer rate-limitation is consequently consistent with the CV curves recorded here with the full-polarisation biofilms (Fig. 4B).

In the absence of polarisation, the microbial development led to heterogeneously scattered colonies, which were far from forming complete coverage of the surface. Both potentially-EA cells and non-EA cells grew in the absence of any significant electrochemical reaction, except the very low currents that control the OCP value. Open-circuit biofilms resulted from the slow multiplication of the sessile cells and they could also benefit from the integration of planktonic cells. Applying the polarisation to such pre-established biofilms made the potentially EA-bacteria shift rapidly to anode-respiring metabolism and grew by using the electrode as electron acceptor. The ET network contained multiple redox systems but was undersized with respect to the number of cells that rapidly turned towards the anode-respiring metabolism. The ET network then developed during polarisation but the multiple redox systems did not optimally match the cell requirements, electron transfer from the cells to the mediators was rate limiting. The structure of the biofilm remained deeply marked by the structure of the initially open-circuited biofilm, probably because the development of the EA cells was hindered by the presence of well-settled non-EA cells.

The delayed-polarisation biofilms provided slightly higher current densities than biofilms formed under full polarisation. The better performance may be due to their less compact structure, which did not limit mass transfers inside them. It has already been observed that small microbial scattered colonies on a cathode surface provide higher currents than biofilms formed by larger

colonies (Pons et al., 2011). Moreover, the full polarisation biofilms made a uniform layer that completely masked the fibres of the cloth electrodes, while delayed polarisation led to microbial settlement around the fibres. A recent study (Pocaznoi et al., 2012) shown that biofilms formed around ultra-microelectrodes (diameter less than 50 μm) give higher current density than biofilms formed on conventional macro-sized electrodes. This positive effect has been attributed to the densification of the electron transfer network due to the particular properties of ultra-microelectrodes (Bard and Faulkner, 2001). Such an ultra-microelectrode effect could also explain the high current density that has been recently reported with fibre electrodes (Chen et al., 2011; He et al., 2011; Wei et al., 2011). Here the fibres of the cloth electrode were around 8 μm in diameter. The biofilms formed under delayed polarisation may have benefited from an ultra-microelectrode effect when they formed around the fibres and kept a micro-structure. In contrast, the thick uniform biofilm prepared by full polarisation was not able of exploiting this positive effect. The two basic explanations (mass transfer limitation and possible ultra-microelectrode effect) converge to identify the biofilm structure as the cause of the higher currents provided by the delayed-polarisation biofilms. The rare studies that have addressed the relationship between the electrochemical performance of a microbial anode and the spatial structure of the biofilm have opened promising tracks. For instance, it has been demonstrated that co-cultures of gram-positive and gram-negative bacteria showed synergistic or mutualistic effect, which corresponded to particular spatial organisation of the mixed biofilms (Read et al., 2010). The co-cultures have resulted in currents two times higher than the single cultures. Controlling biofilm structure to improve the current of microbial anodes should deserve more attention in the future.

This work confirmed the occurrence of an adaptation phase necessary for the cells to develop their EA capacity in response to polarisation as evoked in the introduction section. It also showed the short-time required for the potentially-EA cells to shift to anode-respiring metabolism and distinguished this process from the development of the ET network, which can be longer when polarisation is applied to already formed biofilms.

4. Conclusions

Waiting several days at open circuit before applying polarisation allowed the polarisation duration to be drastically reduced to reach similar current densities. The delayed polarisation gave current density of 9.4 A/m^2 after only 3–9 days of polarisation, while conventional full polarisation required 36 days of continuous polarisation to obtain 6–8 A/m^2. For the first time, this work has shown that applying the polarisation to an established biofilm formed at open circuit may be a promising way to improve the performance of microbial anodes prepared in a natural environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2012.03.042.

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