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Sampling Natural Biofilms: A New Route to Build Efficient Microbial Anodes

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Electrochemically active biofilms were constructed on graphite anodes under constant polarization at -0.1V vs saturated calomel reference (SCE) with 10 mM acetate as substrate. The reactors were inoculated with three different microbial samples that were drawn from exactly the same place in a French Atlantic coastal port (i) by scraping the biofilm that had formed naturally on the surface of a floating bridge, (ii) by taking marine sediments just under the floating bridge, and (iii) by taking nearby beach sand. Current densities of 2.0 A/m^2 were reached using the biofilm sample as inoculum while only 0.4 A/m^2 and 0.8 A/m^2 were obtained using the underlying sediments and the beach sand, respectively. The structure of bacterial communities forming biofilms was characterized by denaturing gradient gel electrophoresis (DGGE) analysis, and revealed differences between samples with the increase in relative intensities of some bands and the appearance of others. Bacteria close related to *Bacteroidetes*, *Halomonas*, and *Marinobacterium* were retrieved only from the efficient EA-biofilms formed from natural biofilms, whereas, bacteria close related to *Mesoflavibacter* were predominant on biofilm formed from sediments. The marine biofilm was selected as the inoculum to further optimize the microbial anode. Epifluorescence microscopy and SEM confirmed that maintaining the electrode under constant polarization promoted rapid settlement of the electrode surface by a bacterial monolayer film. The microbial anode was progressively adapted to the consumption of acetate by three serial additions of substrate, thus improving the Coulombic efficiency of acetate consumption from 31 to 89%. The possible oxidation of sulfide played only a very small part in the current production and the biofilm was not able to oxidize hydrogen. Graphite proved to be more efficient than dimensionally stable anode (DSA) or stainless steel but this result might be due to differences in the surface roughness rather than the intrinsic features of the materials. Finally, a maximal current density of 7.9 A/m^2 was reached with 10 mM acetate after only 8 days of biofilm

formation at -0.1V/SCE . These results are among the best performance values reported in the literature. Using natural biofilms as inoculum should, consequently, be a new, very promising way to rapidly build more efficient microbial electrodes than those produced when the inoculum is drawn from bulk environments.

1. Introduction

The recent discovery of microorganisms able to catalyze the oxidation of organic matters on electrode surfaces has boosted the development of microbial fuel cells (MFCs), which should now be able to exploit a large variety of cheap organic compounds to produce electricity (1, 2). The electrochemically active microorganisms form biofilms on the electrode surface, which oxidize the nutrients and transfer the electrons to the electrode (3). The mechanisms of electron transfer have been demonstrated to be either direct, involving membrane-bound cytochromes for instance, or through natural electron mediators that are produced by the microorganisms and remain entrapped in the biofilm to some extent (4). The involvement of conductive pili has also been demonstrated (5). Several teams have proved that microbial biofilms formed from sediment contain efficient electrochemically active microorganisms able to use graphite anodes as electron acceptors (6, 7).

The efficiency of electricity production in a MFC can be optimized by altering its physical components, such as cell design (4), electrode material (8), or substrate concentration (9–11), and also by improving its biological components. Cho et al. (12) optimized the bacteria themselves by successive cultures of *Shewanella oneidensis* MR-1. Actually the serial cultures implemented with a soluble electron donor (lactate) and acceptor (fumarate) were mainly aimed at preadapting the bacterial metabolism to the anaerobic environment in the MFC. Compared with unadapted bacteria, the anaerobically adapted cells showed improved efficiency in electricity generation of around 30%, even after re-exposure to air. This evoked an adaptation of the microbial population due to metabolic adaptations or genetic mutations (12). Rabaey et al. have described an enrichment by successive transfers of a bacterial consortium from the anodic compartment of an MFC (13). The biofilm formed on the anode was scratched and used to inoculate a new MFC. During the enrichment period, the power output increased from $0.6\text{ W}\cdot\text{m}^{-2}$ of anode surface to a maximum of $4.31\text{ W}\cdot\text{m}^{-2}$. It was concluded that microbial fuel cells enhanced the growth of bacteria that could use the electrode as a final electron acceptor.

The objective of this work was to optimize an anodic electrochemically active biofilm formed from marine environments by sampling at different locations. A biofilm that naturally developed at the surface of a floating bridge was used as the inoculum in a laboratory electrochemical cell. The current provided when the biofilm reconstructed on the surface of the electrode under constant polarization was compared to the current obtained in identical conditions when the cell was inoculated with samples from sediments or beach sand from exactly the same place. Serial substrate additions were also performed with a view to improving the biofilm electrochemical activity. Different anode materials were tested, including graphite, dimensionally stable anode (DSA) (14), and stainless steel.

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2. Experimental Section

2.1. Marine Samples. Natural marine biofilm, marine sediments and beach sand were collected from the Atlantic Ocean (La Tremblade, France). Natural marine biofilm was obtained from a floating bridge in the port of La Tremblade. The biofilm was scraped using a plastic scraper, and around 50 mL of biomaterial was stocked in a glass bottle with 250 mL of fresh seawater for three days at ambient temperature. Sediments were collected a few tens of centimeters below the floating bridge and sand was taken from the beach at the same place. Sediments and sand were stored in closed plastic boxes at ambient temperature. The seawater used in the electrochemical cell was also from La Tremblade.

2.2. Microbial Community Analysis. DNA Extraction from Biofilms. Total DNA was extracted from the microbial suspensions as follows: the microbial cells were suspended in 750 μL of TNE (100 mM Tris-HCl pH 8.0; 50 mM NaCl; 50 mM EDTA pH 8.0) and 50 μL of lysozyme (10 mg/mL). After 10 min at room temperature, SDS 10% (100 μL), sarkosyl 10% (100 μL), and proteinase K (20 mg/mL) (50 μL) were added and the mixture was incubated at 50 $^{\circ}\text{C}$ for 1 h. The cell lysates were further purified after addition of 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), followed by the addition of 1 volume of chloroform:isoamyl alcohol (24:1). The nucleic acids were precipitated by addition of 0.7 volume of isopropanol. The nucleic acids were therefore suspended in a final volume of 50 μL of sterile ultrapure water and frozen at -80°C for long-term storage. For each microbial suspension, five nucleic acids extractions were performed and finally pooled.

Denaturing Gradient Gel Electrophoresis (DGGE) Fingerprinting. 16S rRNA genes fragments were amplified with primers P2 and P3 and analyzed by denaturing gradient gel electrophoresis (DGGE). Briefly, PCR products were loaded in a polyacrylamide gel containing a gradient of denaturant (32–62%) and separated according to their GC content by electrophoresis (75 V, 60 $^{\circ}\text{C}$, 17 h). After electrophoresis, the gel was stained by using a silver nitrate staining as previously described (15).

PCR products were analyzed by DGGE and prominent bands with high relative abundance, and/or bands that were present in some samples but absent from others, were excised and sequenced as previously described (15).

2.3. Experimental Device and Electrodes. Experiments were conducted in 500 mL reactors containing 250 mL of seawater and 250 mL of the marine samples. The reactors were closed hermetically, without any gas flow. A conventional three-electrode system was implemented with a multipotentiostat (VMP2 Bio-Logic SA) interfaced with a computer (EC-Laboratory v.8.3 software, Bio-Logic SA). The working electrodes were graphite (Alfa Aesar, stock no. 10134), dimensionally stable anode (DSA, Electro Chemical Service), and stainless steel 254SMO (Fe 56.1%, Cr 19.9%, Ni 17.8%, Mo 6%, N 0.2%). DSAs are common industrial anodes made of titanium covered with iridium and tantalum oxides. The total exposed electrode area was 25 cm^2 . The electrodes were cleaned before each experiment. Graphite was cleaned by immersion in 0.5 M HCl solution and rinsed gently with distilled water, DSA by 5 h galvanostatic electrolysis at 20 mA/ cm^2 in a 0.1 M sulfuric acid solution, and stainless steel by immersion in 2% HF 0.5 M HNO_3 solution for 20 min followed by rinsing for 1 h with distilled water. The working electrodes were embedded vertically down to the bottom of the reactor and the electric contact was made with a titanium wire. The secondary electrode was a 20 cm^2 surface area platinum grid. All electric potentials were reported against a saturated calomel standard reference electrode (SCE, Radiometer Analytical, TR100) protected with a second porous frit. The potential of the anodes was fixed at -100mV/SCE during

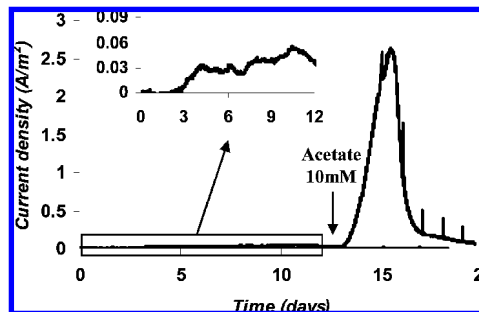


FIGURE 1. Current density versus time with a graphite electrode polarized at -100mV/SCE , inoculation with a natural marine biofilm. Acetate 10 mM was added after day 13.

chronoamperometry. Cyclic voltammetry was performed in situ between -700 and 300mV/SCE at a scan rate of $10\text{mV}\cdot\text{s}^{-1}$.

2.4. Roughness Measurements and Microscopies. Average roughness of the material surfaces was measured with a Zygo New View 100 OMP-0348K white light interferometer monitored with MetroPro software.

Microbial colonization of coupons was verified at the end of the experiments by epifluorescence microscopy or by scanning electron microscopy (SEM). Samples were extracted from the reactors and washed carefully with seawater to remove materials except attached biofilms. For epifluorescent observation, the biofilm was stained with 0.03% orange acridine (A6014, Sigma) for 10 min. The samples were then left to dry in ambient air and 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 processed with the Image-Pro Plus 5.0 software. Scanning electron microscopy (SEM) was carried out on a LEO 435 VP-Carl Zeiss SMT at 10 000 \times magnification working at 10 kV accelerating voltage. Bacteria adsorbed on the electrode surfaces were fixed with 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) applied for 20 min. Then the fixative buffer was poured out and the sample was washed twice for 15 min with fresh cacodylate buffer (0.1 M, pH 7.4) containing 0.2 M sucrose. Postfixation was achieved with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) and 0.15 M sucrose for 20 min. The fixative buffer was then poured out and samples were dehydrated through graded acetone series: 50% acetone for 5 min, 70% acetone for 5 min, 100% acetone for 30 min. Final dehydration was achieved with acetone and hexamethyldisilazane (50:50) until total evaporation under a laminar air flow hood. The samples imaged by SEM were sputtered with a thin layer of gold to guarantee their electrical conductivity. A gold film of approximately 10 nm was grown by means of a Scancoat Six SEM sputter coater (Edwards).

3. Results and Discussion

3.1. Electrochemical Activity with a Natural Marine Biofilm As Inoculum. The graphite anodes were maintained at -100mV/SCE , a potential used in several previous studies dealing with electrode/sediment interfaces (16, 17). The current density variation with time (Figure 1A) showed a first lag period of 3 days, followed by a weak anodic current that increased slowly up to $50\text{mA}/\text{m}^2$ (Figure 1B). During this period, it was suspected that microorganisms progressively colonized the anode surface using endogenous organic compounds as electron donors. The lag period may have been due to the presence of oxygen in the medium during the first days. It has already been suggested that cellular metabolism must be altered in an oxygen limited environment to allow electrogenesis (14, 18). Acetate addition with a final concentration of 10 mM after 13 days boosted the

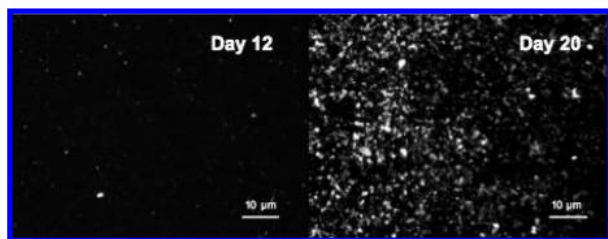
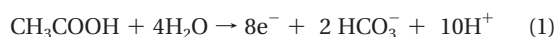


FIGURE 2. Epifluorescence microscopy of graphite anode from the experiment depicted in figure 1, before acetate addition (day 12) or after acetate addition (day 20).

current production and the microbial growth on the electrode surface, as can be clearly seen on the epifluorescence microscopy pictures taken of two graphite electrodes before (day 12) or after (day 20) acetate addition (Figure 2). After reaching a maximal value of 2.6 A/m², the current density decreased rapidly, probably due to the consumption of acetate. The pH decrease from 7.8 to 6.8 may also have contributed to the bacterial growth limitation as described in previous studies (9–11). The microbially assisted electrochemical oxidation of acetate



should result in an acidification of the medium, but it has been claimed that acetate anaerobic digestion, which occurs in parallel because of nonelectrochemically active anaerobic bacteria, participates also in the pH decrease.

Control experiments performed under the same experimental conditions with 500 mL of seawater without biofilm inoculum did not give any current, before or after addition of acetate.

Scanning electron microscopy of several anode surfaces giving current densities from 2.0 to 4.0 A/m² revealed that the electrodes were covered with bacteria, whereas control electrodes that were put in the same reactor but kept on open circuit were not colonized (Figure 3A and B). In Figure 3B and C, the electrochemically active microbes associated with the electrode appeared to be in direct contact with the electrode surface, as has been already observed with biofilms formed in pure culture of *Geobacter sulfurreducens* (5).

3.2. Bacterial Adaptation and Substrate Conversion Rate. With a view to improving the adaptation of the microbial consortium to the electrochemical conditions, serial additions of acetate were made to a batch culture. As previously, the electrochemical cell contained 500 mL of seawater inoculated with the scraped marine biofilm and the graphite electrode was polarized at –100mV/SCE. Acetate was first added after 2 days. The current density showed a fast increase reaching a maximal value of 2.3 A/m² two days after acetate addition (Figure 4). Then, the lack of acetate in the medium resulted in a drop of the current and new additions of acetate, still at a final concentration of 10 mM, allowed the current production to restart. The maximal current densities obtained after each successive addition of acetate were lower but the current production time increased in each case. Integrating the successive peaks gave 1200, 1980, and 3456 C, respectively. Assuming a total transformation of acetate into carbon dioxide (eq 1), as already demonstrated (4), gives eight electrons exchanged per molecule of acetate, and leads to Coulombic efficiencies of 31, 51, and 89% for each successive acetate addition. The part of acetate that was consumed by the electrochemical process increased 3-fold from the first to the third addition. The electrocatalytic properties of the biofilm progressively adapted to the consumption of acetate. It must be noticed that the maximal current density tended to decrease from one acetate addition to the other. It is consequently not likely that the improvement in Coulombic

efficiency was due to an increase of the number of electro-active microbial species or an increase of the efficiency of the species, as such phenomena should lead to an increase of the current density. The improvement in the Coulombic efficiency may be attributed here to gradual selection of the electrochemically active micro-organisms, resulting of a decrease of the nonspecific microbial reactions that consumed acetate. A large part of the acetate was consumed in the first days by competitive microbial processes that vanished as the experiment progressed. A similar effect has been observed by Kim et al. (19) with a study on acetate-fed wastewater MFC. They obtained a 30% increase of the Coulombic efficiency by inhibiting the methanogen micro-organisms that induced the side-consumption of acetate with the addition of 2-bromoethanesulfonate (19).

3.3. Comparison with Other Marine Bioanodes. Remarkably efficient electrochemically biofilms were obtained here by rebuilding a biofilm on a graphite anode from a natural marine biofilm. Were these results due to the presence of a specific microbial population at the location where the samples were taken, or to the method that consisted in scraping a natural biofilm rather than drawing samples from the bulk environment? To settle this issue, similar experiments in which the reactors were inoculated with sediments or beach sand taken from exactly the same place as the marine biofilm were repeated. Sediments were collected a few tens of centimeters below the bridge pillar that the biofilm was scraped from, and sand from the beach in the immediate vicinity. Three electrochemical cells containing each two graphite electrodes (working electrode and control at open circuit) were inoculated in parallel with marine biofilm, marine sediment, and beach sand sampled the same day. The graphite anode was embedded in either sediment or sand with a solid/liquid ratio of 1 (v/v) in each bioreactor. Acetate was added to each bioreactor after 2 days of anode polarization at –100mV/SCE. The lag time was less than 12 h for each microbial source, and a higher current generation started as soon as acetate was added (Figure 5). Current density obtained with marine biofilm increased rapidly to reach a maximal value of 2.0 A/m² after 12 days, whereas marine sediment or beach sand offered only maximal current densities of 0.4 and 0.8 A/m², respectively. The current density values obtained here with sediments and sand were of the same order of magnitude as values reported in the literature for MFC inoculated with sediments. Maximal current densities in the range 0.10–0.15 A/m² were obtained first (6). Current density has been improved with higher concentrations of organic matter. For example, chitin enhanced sediment MFCs offered maximal current densities of 1.20 A/m² (20). In contrast, the current densities obtained here with biofilm inoculum, often higher than 2 A/m², represent the highest values reported.

This result may be compared to the procedure proposed by Rabaey et al., who improved the MFC power output 7-fold during a 2 months enrichment period that consisted of successive cycles of biofilm scratching and inoculation: the biofilm formed on the MFC anode was scratched and further used to inoculate a new MFC (14, 21). It has thus been demonstrated that using anodic biofilms instead of bulk samples is a good way of improving the electrochemical effectiveness of biofilms by successive steps. The results obtained here may be explained in a similar way. Biofilm structuring that occurred naturally on a flat plain surface may have a similar favorable effect to the “scratch and inoculate” strategy implemented by Rabaey et al. The microbial consortia that are naturally structured in biofilm in their environment proved to be very effective in rebuilding effective electrochemically active biofilms in the laboratory. If this fact was confirmed in other environments commonly used to inoculate MFCs, like waste waters or industrial sludge,

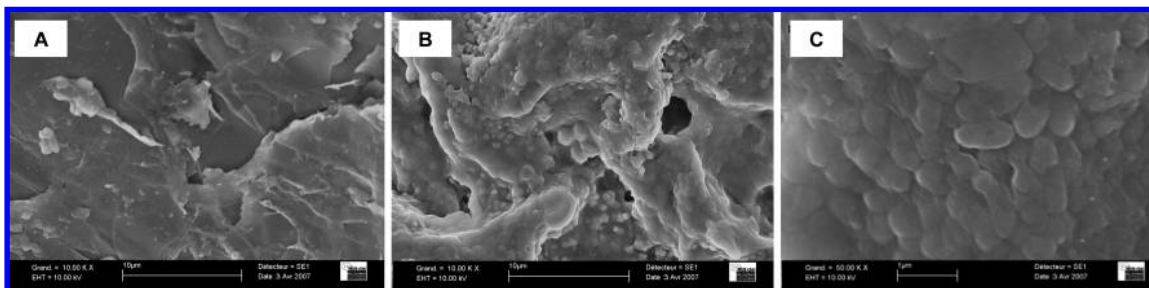


FIGURE 3. Scanning electron micrographs of a control graphite electrode (not polarized) (A) and a working graphite electrode polarized at -100 mV versus SCE for 20 days (B and C), inoculation with a natural marine biofilm. Magnification was set at $10\,000\times$ for A and B and at $50\,000\times$ for C.

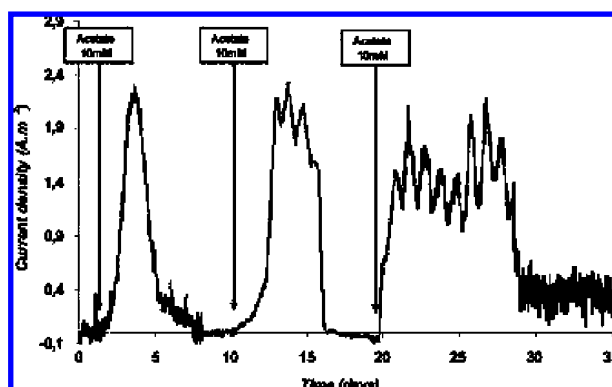


FIGURE 4. Effect of successive additions of acetate on current by a graphite electrode polarized at -100 mV versus SCE, inoculation with a natural marine biofilm.

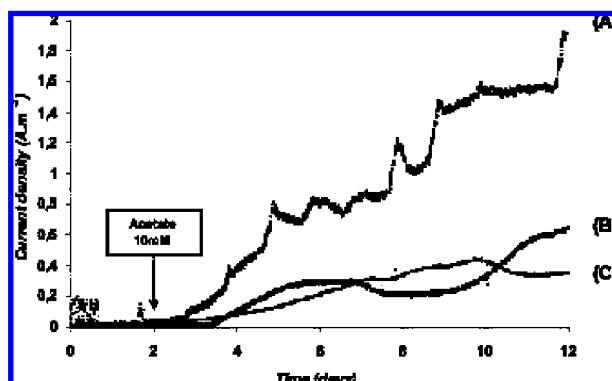


FIGURE 5. Comparison of current density supplied with a working graphite electrode polarized at -100 mV/SCE after inoculation with (A) marine biofilm, (B) beach sand, (C) marine sediment.

it should open up a new route for sampling environmental consortia in a more efficient way: sampling natural biofilms rather than bulk environments.

In sediment MFCs, the oxidation of sulfide on the anode surface has been described as responsible for a significant part of the current production on the anode (4, 22). Sulfide abiotically oxidizes on the electrode producing S^0



that may further be oxidized to sulfate by *Desulfobulbus* species using the anode as the electron acceptor (23). In the experiments reported here, sulfide may result from the reduction of the sulfate contained in seawater by the sulfate reducing bacteria present in the sediment inoculum. In order to examine whether sulfide could be responsible for a part of the current generated here, a cyclic voltammetry (10mV/s) was performed as control with a

clean graphite electrode that was not covered by biofilm in a 10 mM sulfide seawater solution. The 10 mM sulfide concentration that was used was much higher than the sulfate concentration generally encountered in a seawater environment (between 0 and 4.0 mM) (7). The oxidation peak obtained in this control experiment was 10-fold weaker than that obtained with the anode covered by marine biofilm, indicating that abiotic sulfide oxidation can only be responsible for a minor part of the current generated here.

It has been implied that many byproduct of microbial fermentation are abiotically oxidized at the anode surface to provide electrons. Hydrogen has sometimes been suspected of being involved in abiotic oxidation, mainly in single-cell experiments. Actually, hydrogen may be produced on the auxiliary electrode (platinum cathode) and further oxidized on the anode, resulting in a positive artifact (3). To assess the possible occurrence of such an artifact, hydrogen was bubbled through the cell for several hours. No significant current increase was observed, showing that, in our case, the microbial biofilm was not able to catalyze hydrogen oxidation.

3.4. Comparison of Microbial Communities. The structure of bacterial community forming inocula and electrochemically active biofilms were compared with samples coming from (a) the natural biofilm, (b) the electrochemically active biofilm formed from the natural biofilm, (c) sediments, and (d) the electrochemically active biofilm formed from sediments.

DNA extracted from each sample were analyzed by 16 S rRNA-gene-based DGGE. Denaturing gradient gel electrophoresis profiles of DNA from electrodes colonized by natural marine biofilm differed from those of marine biofilm used as inoculum and from those of biofilms derived from sediments or from sediments with the increase in relative intensities of some bands and the appearance of others (Figure 6). These results suggest that the electrochemical efficiency of the marine biofilm was due to certain bacterial populations that are more abundant or exclusive to these biofilms. Interestingly, bacteria close related to *Bacteroidetes*, *Halomonas*, certain species of *Idiomarina*, *Pseudoidiomarina*, and *Marinobacterium* were retrieved only from the most efficient EA-biofilm formed from natural biofilms. Whereas, bacteria close related to *Mesoflavibacter* were predominant on biofilm formed from sediments.

The broad phylogenetic diversity of bacteria known to transfer electrons onto solid phase electron acceptors (24), hints that the diversity of electricity-producing organisms should be considerable. Actually, culture-independent studies, revealed the wide phylogenetic diversity of MFC microbial communities (23, 25–28). The present study revealed that certain bacterial association might be more favorable for electron transfer to anode than others. Future studies unraveling synergetic and antagonistic relationship and trophic interaction between bacteria within biofilm may contribute to explore the mechanism underlying with electron transfer to the anode.

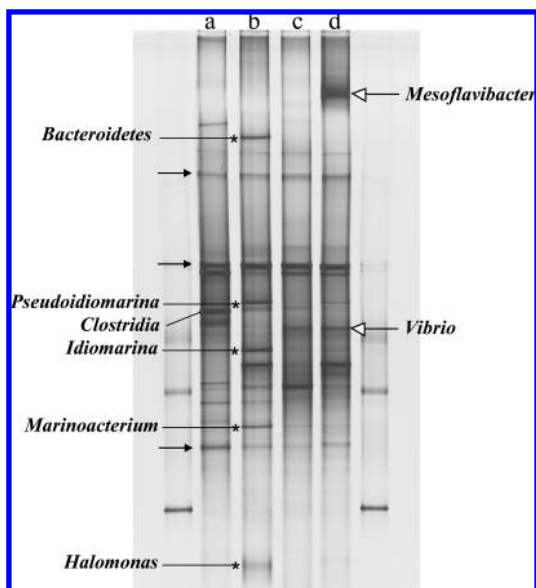


FIGURE 6. DGGE banding profiles of bacterial 16S rRNA genes PCR-amplified from DNA of (a) natural marine biofilm, (b) electrode colonised by natural marine biofilm, (c) marine sediments, (d) electrode colonised by microorganisms from marine sediments. Asterisks indicate bacteria that are more abundant or exclusive to marine biofilm electrode. Filled-head arrows indicate common bacterial populations. Empty-head arrows indicate bacterial population specific to sediments.

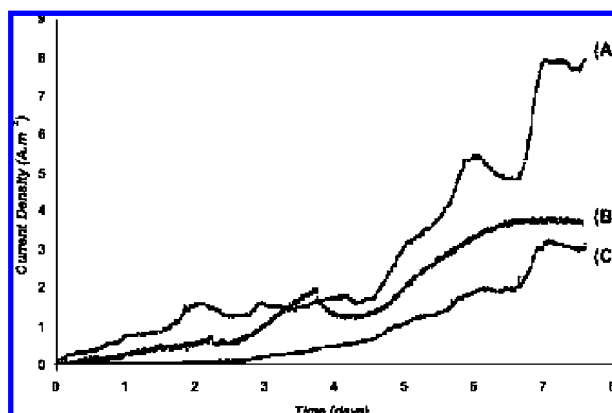


FIGURE 7. Comparison of current density supplied by graphite electrode (A), DSA electrode (B), and 254 SMO stainless steel electrode (C) polarized at -100 mV/SCE, inoculation with a marine biofilm.

3.5. Effect of Anode Materials. Many studies have reported the impact of different anode materials or different surface modifications on the MFC performances (9, 12, 29–31). For example, various chemically modified graphite anodes implemented in sediment MFCs have revealed kinetic activities between 1.5- and 2.2-fold those of plain graphite (32).

Three electrode materials: graphite, dimensionally stable anode (DSA), and stainless steel, were tested following the same procedure at the same time and with the same inoculums, in order to avoid any discrepancy in the sampling and operating conditions. Acetate was added to each reactor after 2 days' incubation with 10 mM final concentration. Higher current density was generated with the graphite electrode; a maximal value of 7.9 A/m² was obtained after 8 days of biofilm growth (Figure 7). The maximal currents with DSA and stainless steel electrodes were 3.8 and 3.1 A/m² respectively. The average surface roughness Ra of the three materials measured with a light interferometer gave Ra = 5.6

μm for graphite, Ra = 3.2 μm for DSA, and a 19-fold lower value Ra = 0.3 μm for stainless steel. It is commonly agreed that higher surface roughness as specific materials properties favors microbial adhesion, when the values are of the same order of magnitude as the bacterial cell size (33). It has been observed with a pure culture of *Geobacter sulfurreducens* that the current supplied under constant polarization on graphite and DSA anodes is directly proportional to the surface roughness of the electrode (34). Here current density and surface roughness were also not far from proportionality for graphite and DSA. It can be suggested that increasing the surface roughness of stainless steel might greatly favor the microbial adhesion and consequently increase the current density obtained. Assuming a linear relationship, stainless steel might supply up to 57 A/m² if its surface roughness was increased from 0.3 to 5.7 μm (same value as graphite). This means it might be 7 times as efficient as graphite. This expectation is certainly overestimated, but stainless steel cannot be rejected on the sole basis of the raw current densities recorded here. Experiments are now in progress to compare materials with similar surface morphology.

Acknowledgments

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