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Electroactive biofilms: new means for electrochemistry

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Abstract

This work demonstrates that electrochemical reactions can be catalysed by the natural biofilms that form on electrode surfaces dipping into drinking water or compost. In drinking water, oxygen reduction was monitored with stainless steel ultra-microelectrodes under constant potential electrolysis at −0.30 V/SCE for 13 days. 16 independent experiments were conducted in drinking water, either pure or with the addition of acetate or dextrose. In most cases, the current increased and reached 1.5–9.5 times the initial current. The current increase was attributed to biofilm forming on the electrode in a similar way to that has been observed in seawater. Epifluorescence microscopy showed that the bacteria size and the biofilm morphology depended on the nutrients added, but no quantitative correlation between biofilm morphology and current was established. In compost, the oxidation process was investigated using a titanium based electrode under constant polarisation in the range 0.10–0.70 V/SCE. It was demonstrated that the indigenous micro-organisms were responsible for the current increase observed after a few days, up to 60 mA m^{-2}. Adding 10 mM acetate to the compost amplified the current density to 145 mA m^{-2} at 0.50 V/SCE. The study suggests that many natural environments, other than marine sediments, waste waters and seawaters that have been predominantly investigated until now, may be able to produce electrochemically active biofilms.

1. Introduction

A microbial biofilm is a complex three-dimensional structure in which micro-organisms grow embedded in the matrix of the extracellular polymeric substances they produce [1]. Biofilms develop on all kinds of surfaces such as ship hulls, off-shore units, pipes for oil transportation or water distribution, stones, clothes, plant roots and leaves, human skin, etc. It is has long been known that natural biofilms are able to increase both aerobic [2, 3] and anaerobic [4] corrosion by catalysing several electrochemical reactions on metal surfaces. Biofilms are thus responsible for so-called biocorrosion in an extremely wide variety of industrial sectors, leading to significant financial losses [5–7]. For instance, the microbially influenced pitting corrosion of heat exchanger tubing in nuclear power plants (Ontario Hydro of Canada) has been estimated to cost the corporation $300,000 per unit per day in replacement costs. Escom, the national power utility of South Africa that provides 90% of power requirements for that country, has detected microbially influenced corrosion (MIC) of carbon steel in cooling water systems in virtually all their power plants. The costs associated with repairs and down time are millions of dollars annually [8].

In addition, recent studies have highlighted the unexpected ability of biofilms to achieve direct electron transfer with electrodes [9–11]. It has been shown that a fuel cell composed of a graphite anode embedded in marine sediment and a graphite cathode in the overlying seawater generates electrical power *in situ* that increases as the biofilm grows [10]. The graphite electrode buried in the sediments was found to be colonised by *Geobacteracea* bacteria that oxidised acetates contained in the sediments and transferred the electrons to the anode. *Geobacter metallireducens* [12, 13] and *Geobacter sulfurreducens* [14] revealed their remarkable ability to use the graphite anode as sole electron acceptor. The most important discovery was that the electrons from acetate oxidation were transferred directly to the graphite electrode, without an electron-transfer mediator [15]. Numerous so-called microbial fuel cells have therefore been proposed either using natural microbial consortia or with pure cultures of different strains [16–19]. Many micro-organisms other than *Geobacter* have been identified which are also able to catalyse the oxidation of organic compounds like acetates or sugars on graphite anodes [19, 20]. *Shewanella putrefaciens* has been shown to be able to oxidise lactate following the same direct electron transfer pathway to a graphite anode [21]. It has been stated that *Rhodoferax ferrireducens*, isolated from

aquifer sediments, metabolises glucose and other sugars into CO_2, transferring the electrons directly to a graphite electrode without the presence of an electrochemical mediator [22]. Other bacteria such as *Aeromonas hydrophila* [23] and *Geothrix fermentans* [24] have also been found to have electroactive properties. Different mechanisms have been proposed to explain bacterial electron transfer to anodes [18, 25]. Four mechanisms of electron transfer have thus been distinguished in microbial fuel cells [26]:

(i) abiotic oxidation on the electrode of the reduced compounds produced by the microbial fermentation,
(ii) enhanced electron transfer with artificial mediators (benzylviologen, thionine, neutral red, 2,6-dichlorophezнolindophenol, etc.) added to solution,
(iii) micro-organisms that produce their own mediators,
(iv) direct electron transfer without a mediator, either artificial or microbially produced.

The latest investigations on *Geobacter sulfurreducens* have shown that electron transfer to flat massive graphite anodes or dispersed Fe(III) oxides may involve outer membrane c-type cytochromes [25] or conductive pili that act as external nanowires [27]. To date, only a few studies have dealt with similar cathodic processes. Microbial catalysis of a cathode reaction has been demonstrated with the reduction of fumarate on a graphite cathode catalysed by direct electron transfer through *Geobacter sulfurreducens* [28]. A second example concerns natural seawater biofilms catalysing the reduction of oxygen on stainless steel [29].

Up to now, almost all studies have been carried out in sea and ocean environments (water and sediments) and in wastewater. These two environments have been the source of most of the electrochemically active bacteria that have been identified and isolated since 2002. The purpose of the present study was to explore the capacity of other natural environments to produce biofilm-driven electrochemical catalysis. This paper reports an investigation carried out in two different natural environments, compost and drinking water. Compost was chosen as a kind of soil containing a rich microbial ecology and drinking water was investigated because of its economical and societal importance. Biofilms formed in compost were checked for catalysis of the oxidation of organic compounds, similar to the processes identified in sediments. Biofilms formed in drinking water were checked for the catalysis of oxygen reduction, because the biofilm-driven catalysis of oxygen reduction has been widely studied in the area of aerobic biocorrosion of stainless steels in seawater and other kinds of water environments like rivers and waste water treatment plants [2, 3, 30–34]. Most of the field studies were conducted using only galvanic coupling of two electrodes, without controlling the electrode potential during the day-long experiments [29, 35, 36]. Using compost and drinking water allowed experiments to be easily carried out in the laboratory with potential control through a three-electrode system.

2. Materials and methods

2.1. *Set-up for drinking water*

Four 3 l volume tanks were fed in parallel with drinking water directly from the city distribution network. No dechlorination was performed before feeding the tanks. Feeding for 1 h per day at 5 l h^{-1} was assumed to achieve complete refilling every day. The temperature of each tank was controlled at 25 °C with a double cell wall. Micro-sensors were composed of five AISI 316 stainless steel wires 0.05 mm in diameter (Goodfellow) incorporated in an insulating epoxy resin (Struers) and connected together to a coaxial cable. The final micro-sensors had an external diameter of 5 cm where only the five ultra-microelectrodes of 0.05 mm diameter each were electrochemically active. The micro-sensors were polished on a P4000 disk (LamPlan polishing Technology) before each experiment. They were immersed only a few centimetres from the water/air interface, where the concentration of dissolved oxygen was assumed to be constant. A 0.5-mm diameter platinum wire was used as the counter electrode. Potential was maintained for several days at − 0.30 V vs. a saturated calomel reference electrode (SCE) on four independent channels of a multi-potentiostat (VMP1 Bio-Logic SA) interfaced to a computer with the EC-Lab v.8.3 software package (Bio-Logic SA). Voltammetry was performed with the same experimental set-up.

2.2. *Set-up for compost*

A 3 l tank was filled with biological compost (Eco-Terre) purchased from a garden centre. Because of the variability of the conductivity of compost from one sample to another, a 10 mM NaCl solution was added to fill the tank to the upper surface of the compost. A titanium-based working electrode of 2.5 × 10 cm surface area was placed vertically in the wet compost so that a surface area of around 20 cm^2 was in the compost. A 1.27-cm diameter graphite rod, buried more than 20 cm deep, was used as the counter electrode. Electrochemical measurements were performed with a multi-potentiostat (model VMP2 Bio-Logic SA) interfaced to a computer with the EC-Lab v.8.3 software (Bio-Logic SA). Before each experiment, the working electrodes were cleaned by 5-h galvanostatic electrolysis at 20 mA m^{-2} in 0.1 M sulphuric acid.

2.3. *Epifluorescence microscopy*

The biofilms were stained with a fluorescent dye solution of 0.03% acridine orange (A6014, Sigma). Acridine

orange is absorbed by bacteria and its fluorescence under 450–490 nm light illumination made it possible to observe the biofilm easily. Micro-sensors from drinking water and electrodes from compost were dipped in the dying solution for 10 min. The electrodes were then dried in air and analysed using an objective (50×) on a Carl Zeiss Axiotech 100 microscope equipped for epifluorescence with an HBO 50/ac mercury light source and the Zeiss 09 filter (excitor AP 450-490, reflector FT 510, barrier filter LP 520). Images were acquired with a monochrome digital camera (Evolution VF) interfaced to a computer with the Image-Pro Plus v.5 software.

3. Results

3.1. *Electrochemically active biofilms in drinking water*

Voltammograms were recorded at 1 mV s^{-1} from 0.0 to −0.70 V/SCE in drinking water coming from the tap without preliminary treatment or addition, with four different micro-sensors. Each micro-sensor was composed of five stainless steel ultra-microelectrodes 0.05 mm in diameter. Experiments were performed in aerated drinking water and in deoxygenated (20 min nitrogen bubbling) water. In this case, the nitrogen flow was maintained continuously over the solution. Comparison of the curves in Figure 1 confirms that the micro-sensors gave current–potential curves which corresponded to the slow electrochemical reduction of dissolved oxygen on stainless steel:

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \qquad (A6)$$

The differences observed in the current values, from 4 nA (micro-sensor 17) to 10 nA (micro-sensor 16) at − 0.70 V/SCE in anaerobic conditions, were probably due to the poor control of the real active surface area of the five microelectrodes. Because of their small size (diameter 0.05 mm), the stainless steel wires may not have been exactly perpendicular to the external surface of the sensor, or resin may have masked a part of the wires. The resulting cross-sectional surface area of the five wires may consequently have varied from one micro-sensor to another. For all micro-sensors, oxygen started to be reduced at around −0.30 V/SCE. This value was chosen for the chronoamperometry experiments because it made the reduction of oxygen possible on the stainless steel material and the kinetics remained very low. These are the best conditions to bring to light a possible catalysis of the reaction.

Chronoamperometry experiments were carried out with four micro-sensors dipping in four independent 3 l tanks and polarised at −0.30 V/SCE for several days. Each tank was refilled each day with drinking water. One tank contained pure drinking water. To accelerate biofilm formation, nutrients were added in the three other tanks every day, just after the refilling operation: acetate 5 mM, acetate 10 mM or dextrose 2.8 mM. After 13 days polarisation, micro-sensors were analysed by epifluorescence microscopy. Then, each micro-sensor was cleaned with ethanol and polished before being replaced in a water tank different from the tank where it has been dipping previously. Four successive experiments were achieved.

In the first and second experiments (Figure 2) the current maintained its low initial value for several days before it increased slowly. In the fourth experiment, the current increased regularly from the first day of exposure. It is possible that contamination of the set-up favoured the fast appearance of electrochemically active biofilms. The current jumps observed every 24 h corresponded to tank refilling. The sinusoidal evolution of

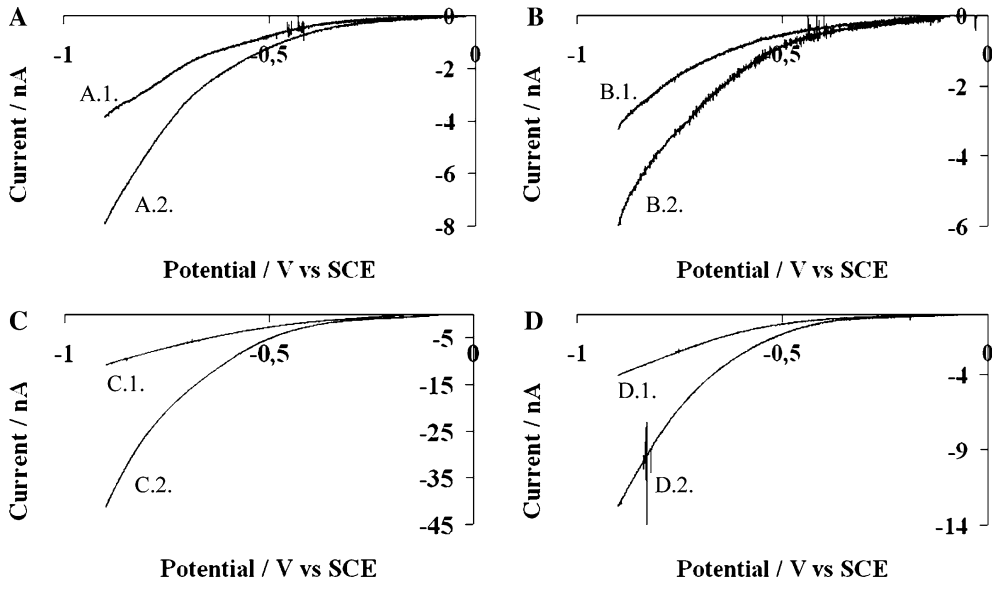

Fig. 1. Voltammograms of four different micro-sensors numbered 12 (graph A), 14 (graph B), 16 (graph C) and 17 (graph D) in anaerobic (A.1.; B.1.; C.1.; D.1.) and aerobic (A.2.; B.2.; C.2.; D.2.) conditions; scan rate 1 mV s^{-1}.

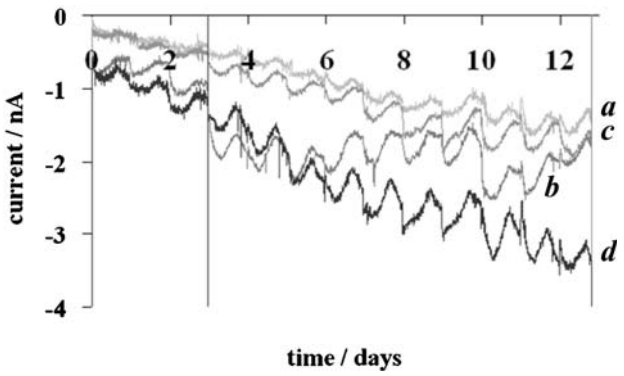

Fig. 2. Chronoamperometry at −0.30 V/SCE with four micro-sensors in parallel in drinking water alone (curve *a*), or supplemented with acetate 5 mM (curve *b*), acetate 10 mM (curve *c*), or dextrose 2.8 mM (curve *d*).

current fitted the evolution of temperature in the tank. The double cell wall was actually not efficient enough to stabilise temperature inside the tanks, mainly because of the daily feeding with fresh water. The current increases observed in 15 experiments and expressed by the ratio of the final current on the initial current are reported in Table 1. This factor varied from 1.5 to 9.5 without any obvious link with the sensor itself or the nutrients added.

After 13 days' polarisation, the micro-sensors were observed by epifluorescence microscopy. For each ultra-micro-electrode, two pictures were taken, which gave 10 pictures for each sensor. On any given sensor, all 10 pictures generally presented very similar morphology of the biofilm. Figure 3 shows pictures obtained on the four sensors of the fourth experiment, which are representative of the different biofilm morphologies. Biofilm formed on all the sensors with remarkable differences in morphology, depending only on the nutritive conditions. For a defined nutritive condition, the biofilm aspect was similar in all experiments (Table 1). Scattered small colonies were obtained in drinking water alone, large colonies when water was supplemented with 5 mM acetate or 2.8 mM dextrose, and rather uniform biofilm in the presence of 10 mM acetate. The size of each individual bacterium also depended on the composition of the medium as reported in Table 1. The nutrient conditions controlled the bacteria size and the biofilm aspect but no clear correlation appeared between the biofilm morphology and current density recorded.

3.2. *Electrochemically active biofilms in compost*

Four different tanks were filled with 3 l of compost. Because of the variability of the conductivity of compost from one sample to another, a 10 mM NaCl solution was added to fill the tank up to the upper surface of the compost. A different potential value (0.10, 0.40, 0.50 and 0.70 V/SCE) was imposed in each tank. The current remained very low for at least one day as expected in the absence of any electrochemical process. After the initial lag period, the current increased with time until a poorly defined levelling-off was reached. The results gathered in Table 2 give the average current density reached at this plateau. The maximum current was obtained for $E = 0.70$ V/SCE.

Figure 4A shows a typical variation of the current during polarisation at 0.50 V/SCE. The current density increased slowly from the first day, reached a maximum of around 60 mA m^{-2} after 3 days and then stabilised at around 40 mA m^{-2}. On the 6th day, sodium acetate was added to the medium to obtain a 10 mM final concentration. This nutrient supply provoked a linear increase of the current density, which reached 80 mA m^{-2} after 8 days. The experiment was repeated with acetate added to the compost initially. The current density increased regularly after the 2-day initial lag period up to 145 mA m^{-2} (Figure 4B). The same procedure was repeated with compost that had been previously sterilised by 20 min autoclaving at 121 °C. Curve 2 in

Table 1. Current measurements, bacteria size and biofilm morphology on the microelectrodes at the end of the polarisation at −0.30 V/SCE

Experiment	Medium	Sensor	Current (*x*-fold)	Bacteria size[a]	Biofilm aspect
1	Drinking water	17	15	−	Scatterred small colonies
	+ Acetate 5 mM	16	3.8	+ +	Large colonies
	+ Acetate 10 mM	12	9.5	+ +	Uniform
	+ Dextrose 2.8 mM	14	5.3	+	Large colonies
2	Drinking water	14	2.8	−	Scatterred small colonies
	+ Acetate 5 mM	17	1.7	+ +	Large colonies
	+ Acetate 10 mM	16	1.5	+ +	Uniform
	+ Dextrose 2.8 mM	12	2	+	Large colonies
3	Drinking water	12	3.6	−	Scatterred small colonies
	+ Acetate 5 mM	14	2.3	+ +	Large colonies
	+ Acetate 10 mM	17	7.3	+ +	Uniform
	+ Dextrose 2.8 mM	16	3.4	+	Large colonies
4	Drinking water	17	–	−	Scatterred small colonies
	+ Acetate 5 mM	16	2.4	+ +	Large colonies
	+ Acetate 10 mM	12	1.5	+ +	Uniform
	+ Dextrose 2.8 mM	14	4	+	Large colonies

[a]Bacteria size: small (−), standard (+), large (+ +).

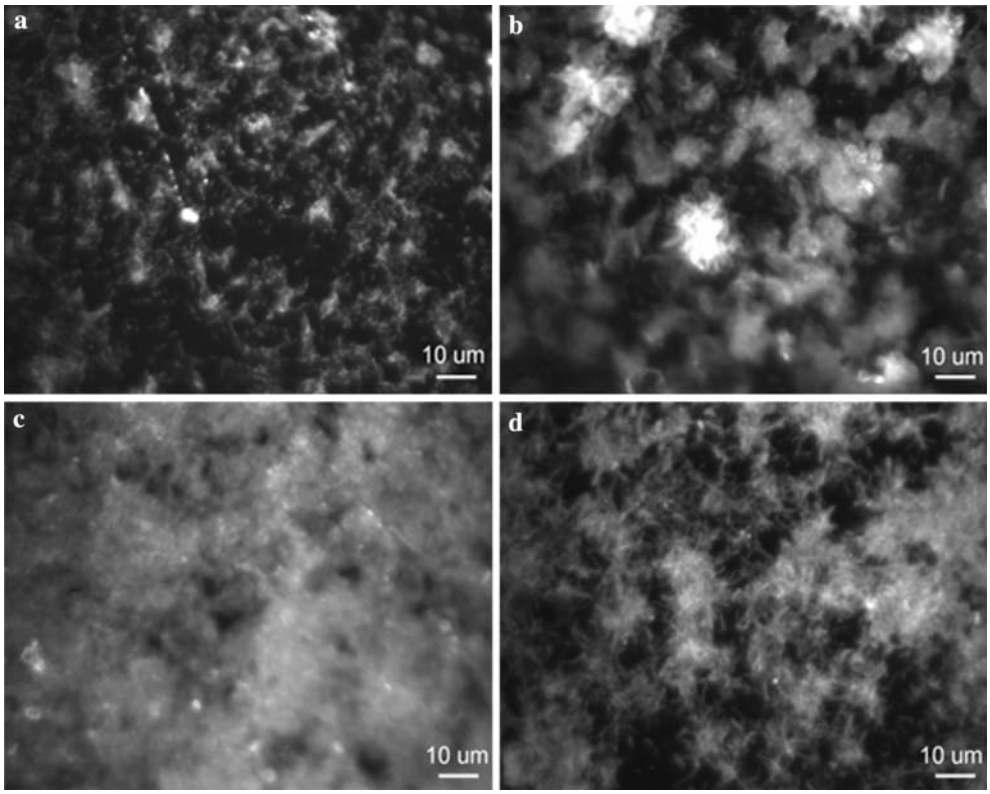

Fig. 3. Epifluorescence microscopy of the ultra-microelectrodes of after 13 days chronoamperometry (experiment no. 4); drinking water alone (a), supplemented with acetate 5 mM (b), acetate 10 mM (c), or dextrose 2.8 mM (d).

Figure 4A shows that there was no current increase after 7 days in this case. On the contrary, the low current that was observed for 4 days vanished and remained very close to zero for the rest of the time. This low initial current, which was observed reproducibly in all experiments performed after autoclaving the compost, was certainly due to reduced compounds that formed during autoclaving. Moreover, adding 10 mM acetate on the 7th day did not cause any change in the low current recorded.

Comparing the results obtained in compost with or without preliminary sterilisation clearly demonstrated that the initial presence of micro-organisms in the compost was necessary for an oxidation process to occur. This assumption is correlated with microscopic analyses, which showed the presence of a biofilm on the electrode surface (Figure 5). The effect of acetate addition showed that acetate can be an excellent electron donor of the oxidation process.

4. Discussion

The biofilm-driven catalysis of oxygen reduction on stainless steels in seawater and different natural waters is well documented [2, 3, 30–34]. Several mechanisms have been proposed to explain this catalysis. The production of hydrogen peroxide by the biofilm has been assumed to play a key role [32, 37], modification of the oxide layers on stainless steel beneath the biofilm [38] may have an effect, involvement of extracellular compounds and enzymes [39–42] has also been proposed, and the preponderant involvement of manganese ions and manganese oxidising bacteria has been claimed [43]. It may be assumed that the actual mechanism depends on the nature of the environment, the nature of the stainless steel and the operating conditions, and that it may be the result of a combination of the different fundamental reactions that have been identified. Nevertheless, all studies agree that the electrochemical phenomena are directly correlated to biofilm growth on the material surface. It may be reasonably assumed that the same biofilm-driven catalysis of oxygen reduction occurred here in drinking water, even though no obvious correlation has been established yet between the biofilm morphology or the surface coverage ratio and the current recorded. These preliminary experiments show that the presence or absence of nutrients in drinking water has a significant influence on the morphology of the biofilms. Drinking water is an easy-to-handle medium that should allow further investigation in the laboratory under well-controlled conditions.

The biofilm-driven catalysis of oxygen reduction on stainless steel has been used to design amperometric biofilm sensors that have been successfully applied to seawater. Such sensors are based on the galvanic

Table 2. Current densities obtained in compost according to the working electrode potential

Working electrode potential/V *vs.* SCE	0.10	0.40	0.50	0.70
Average current density/mA m^{-2}	50	25	55	75

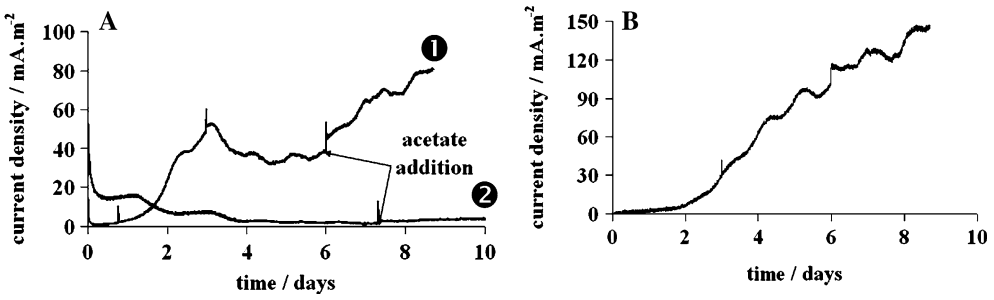

Fig. 4. (A) 1 – Chronoamperometry in compost + NaCl 10 mM at 500 mV/ECS. Acetate 10 mM was added on the 6th day. 2 – Chronoamperometry in sterilised compost polarised at 500 mV/ECS. (B) Chronoamperometry in compost + NaCl 10 mM + acetate 10 mM at 500 mV/ECS.

coupling through an electrical resistance of a stainless steel electrode and an electrode of a different material (e.g., zinc) that plays the role of the anode. The biofilm-driven catalysis of oxygen reduction on the stainless steel electrode induces a current increase, which is measured as the voltage drop at the electrical resistance. It has been demonstrated that the current increase is directly linked to the very first stage of biofilm formation. Because of its simplicity and robustness, this sensor seems perfectly suited for early in-line detection of biofilm formation in seawater environments. An attempt has been made to adapt the principle to waters of low ionic conductivity [44]. In this case, the current was maintained at low values by choosing a high electrical resistance between the coupled electrodes. In the present work, for the first time to our knowledge, it was proposed to detect the catalysis of oxygen reduction in drinking water under strict potentiostatic conditions, i.e., under constant potential and without any limit on the current recorded, using the micro-electrode technique. These first results seem very promising for the design of an amperometric sensor for biofilm detection on the basis of micro-electrode technology. According to the microscopic images presented, the sensor should be able to detect the very first stage of biofilm formation.

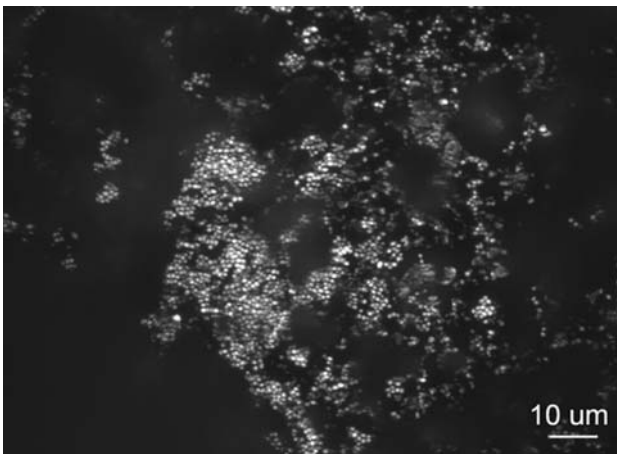

Fig. 5. Epifluorescence microscopy of the working electrode surface after 9 days' polarisation at 0.5 V/SCE in compost (experiment of Figure 3A-1).

The oxidation process implemented in compost gave current densities of the same order of magnitude as those reported in the literature for anodes embedded in marine sediments, about 100 mA m^{-2} [10], or even higher when acetate was added. Acetate is known to be an electron donor for many micro-organisms. Acetate has thus been identified as the substrate of *Geobacter* species that produce electricity by transferring electrons to graphite anodes [14]. The microscopic analysis showed that biofilm coverage was very far from complete. It may consequently be believed that the current produced could easily be enhanced by improving biofilm growth on the electrode surface. Work is now in progress to isolate and identify the micro-organisms that are responsible for the catalytic effect of acetate oxidation.

5. Conclusions

This study widens the scope of results that have been obtained over several years on the microbial catalysis of electrochemical reactions. Up to now, marine sediments and wastewaters were the privileged environments of most studies. The results obtained here with standard compost and drinking water tend to demonstrate that the electrochemical proprieties of biofilms may be much more widespread than expected until now. Even poor environments, such as chlorinated tap water, may produce efficient electrochemical biofilms. A new field of investigation is opening up for bioelectrochemistry.

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