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Electrochemical activity and bacterial diversity of natural marine biofilm in laboratory closed-systems

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ABSTRACT

Even if a widely shared mechanism actually does not exist, it is now generally accepted that, in aerobic conditions, marine electrochemically active biofilms (MEABs) induce faster oxygen reduction on stainless steel immersed in seawater. This phenomenon has been widely studied, but nearly all the experiments found in literature have been conducted in open-systems (i.e. experimental environments where seawater is constantly renewed). In this work we tried to obtain, in open circuit and potentiostatic conditions, MEABs in different laboratory closed-systems without water renewal (mesocosms), in order to verify the relationship between electrochemical activity and biofilm composition. The diversity of the microbial populations of biofilms obtained by our new kind of approach was examined by the DGGE technique (denaturing gradient gel electrophoresis). MEABs were obtained in all the mesocosms from 2000 to 2 L, showing in some cases electrochemical performances comparable to those of open-systems, and a very high genetic variability. Our DGGE results underline the difficulty in finding a correlation between electrochemical activity and composition of microbial populations.

1. Introduction

It is well known that active–passive alloys (e.g. stainless steel), in natural seawater, undergo a shift of open circuit potentials (OCP) towards noble direction (ennoblement), increasing the probability of localized corrosion attacks. This behaviour in marine aerobic seawater was experimentally correlated to the presence of biofilm more than 30 years ago [1]. Between the eighties and the nineties much research was performed on this topic.

Nowadays, some important evidences on ennoblement are:

1) after biofilm settlement, OCP can be shifted up to ≅350 mV vs. SCE [2];
2) this phenomenon is widespread, being observed in different parts of the world (Gulf of Mexico [3], Baltic Sea [4], Mediterranean Sea [5], Arabian Gulf [6], China [7]);
3) the OCP ennoblement is not limited to seawater, but it regards fresh water as well [8].

The mechanism that causes ennoblement clearly is a very important topic.

Even if a widely shared mechanism actually does not exist, it is now generally accepted that OCP ennoblement in aerobic seawaters is related to an accelerated rate of electron transfer from metal to an external acceptor.

In marine environments ennoblement has been attributed to depolarization of the oxygen reduction reaction due to acidification of electrode surface [9,10], electrochemical reduction mediated by the activity of manganese oxidizing bacteria [11–13], oxygen reduction catalysis by organometallic compounds [14], microbial enzymes [2,5,15] and the effects of changes in hydrogen peroxide conformation [16].

Two recent reviews [17,18] cover the remarkable hypotheses on kinetic mechanisms (faster electron transfer mediated by biopolymer metal complexes, enzymes, manganese oxidizing bacteria metabolism) and on thermodynamic mechanisms (changes in O_2 E_{eq} with pH and p_{O2} alterations) along with an H_2O_2 mediation mechanism. An enzymatic hypothesis is discussed in a monographic review [19].

Although the mechanism by which the biofilm causes "cathodic depolarisation" is still not ascertained, the biofilm's capability of accelerating electron transfer from metal to an external acceptor (cathodically) and towards metal from an external donor (anodically) has been exploited in microbial fuel cells (MFCs). In these systems the chemical energy of oxidable substrates can be converted in electrical

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energy employing electrochemical bacterial activity [20–22]. From this point of view, ennoblement occurring in natural marine environments would constitute the cathodic compartment of an MFC. Biofilm-driven catalysis may be a promising track to design new low-cost cathodes for polymer electrolyte membrane (PEM) fuel cells [20] and the use of marine biofilms could greatly improve the cathodic reaction performance of MFCs [23,24].

The catalysis of oxygen reduction on metallic materials has been widely studied in the domain of aerobic corrosion, but, in literature, nearly all the studies on marine aerobic seawater describe experiments conducted in open-systems, i.e. experimental environments where seawater is constantly renewed [3,5–7]. Just one study performed in a laboratory semi-closed system (overall hydraulic retention time of 24 h) has been found [4].

Research on the microbial composition of these marine electrochemically active biofilms (MEABs) is mainly focussing on benthic MFCs, in which the anode is embedded in anoxic marine sediments while the cathode is placed in the overlying aerobic seawater [25].

Several studies have already investigated the biodiversity of biofilm grown on the surface of anodes [26–28] and cathodes [29–32] in marine environments, showing that these MEABs reveal a large microbial diversity and that, at least for marine aerobic biofilms, their electrochemical activity is not linked to the preponderant presence of specific genera or species. Microorganisms involved in electrochemical activity may vary according to the environment, and bacterial communities present on cathodes are phylogenetically diverse.

In the work presented here we tried to obtain the development of MEABs in different laboratory closed-systems without water renewal (defined in this work as "mesocosms", for the detailed description see Materials and methods). The electrochemical activity was monitored measuring the OCP signal [4–7,12] and the current densities when a constant cathodic potential was imposed [33,34].

Furthermore, the diversity of the microbial populations of biofilms (electrochemically active and not active) obtained by our mesocosm approach was also examined by the DGGE technique (denaturing gradient gel electrophoresis), in order to obtain new data on MEAB biodiversity.

The main questions investigated here are:

1) is it possible to reproduce MEAB in different scale mesocosms, in open circuit and potentiostatic conditions?
2) what is the genetic similarity degree of MEAB populations obtained in different mesocosms?

2. Materials and methods

2.1. Samples and connections

For all the tests we used stainless steel samples 25 mm × 10 mm large, 1 mm thick, cut from a plate of a commercial super-austenitic alloy (UNSS 31254: Cr = 19.5–20.5%, Ni = 17.5–18.5%, Mo = 6–6.5%, N = 0.18–0.22%, Cu = 0.5–1%, S < 0.01%, Si < 0.8%, P < 0.03%, Mn < 1%, C < 0.02%, Fe = balance), drilled (Ø = 1.6 mm), screwed, and then treated with emery papers up to P1200. Before the immersion samples were cleaned with HCl 0.1 N. Electrical connections between SS samples, immersed in the solution, and wires outside were realized through titanium rods (Ø = 2 mm) suitably screwed at one hand. Titanium rod surface exposed to seawater was a negligible fraction of connected SS specimen surface ($S_{Ti}/S_{SS} < 5\%$).

2.2. Biofilm covering evaluation

SS samples (from each different experimental set-up and condition) were removed from the test tanks, during or at the end of each experiment, gently rinsed in seawater sterilized by filtration (Millipore, 0.22 μm pore size) in order to remove unattached cells, fixed with 2% paraformaldehyde solution for 30 min, and washed in filtered phosphate buffer saline (PBS). Samples were stored at 4 °C in PBS, before staining and microscopic analysis. After staining of bacterial cells with DAPI (4'-6-diamidino-2-phenylindole, Sigma) [35], samples were observed at 400× magnification using an Olympus BX41 epi-fluorescence microscope coupled with an UV filter block for DAPI. A digital camera CAMEDIA 5060 (Olympus) was used to acquire 30 images of 67,500 μm^2 each, randomly chosen on the SS surface of each sample. Images were transformed to tiff format (RGB colour) and the surface fraction covered by bacteria was measured by means of "Image J" software [36].

2.3. DGGE analysis

At the end of each experiment, once a stable electrochemical signal plateau was reached, biofilms were removed from the SS samples by sonication (90 s) (Branson3200TM) in 30 ml 0.85% NaCl. After direct extraction from cell suspensions, DNA was checked for quantity and quality, by optical density measurements and agarose gel electrophoresis, respectively.

Cell suspensions were centrifuged at 13,000 rpm for 15 min. The resulting cell pellets were suspended in 750 μl of TNE (100 mM of Tris–HCl pH 8.0; 50 mM of NaCl, 50 mM of EDTA pH 8.0) and 50 μl of 10 mg/ml lysozyme solution were added. After 10 min incubation at room temperature, 0.5 g of glass beads were added and cells were lysed by bead beating (3 times 30 s at 1.25 rpm) (RetschTM). Subsequently, 100 μl of SDS and 100 μl of sarkosyl were added to the tube. Proteinase K (50 μl of 20 mg/ml solution) was added, followed by incubation at 50 °C for 1 h. Afterwards, 1 volume of phenol: chloroform:isoamylalcohol (25:24:1) was added, mixed thoroughly and centrifuged for 10 min at 13,000 rpm. Subsequently, the supernatant was transferred to a new Eppendorf tube and 1 volume of chloroform:isoamylalcohol was added. The suspension was mixed thoroughly and centrifuged for 10 min at 13,000 rpm. The DNA present in the aqueous phase was precipitated by addition of 0.8 volumes of isopropanol followed by centrifugation at 13,000 rpm. The DNA pellet was resuspended in 50 μl of water and stored at −80 °C.

DNA was extracted as described above and the V3 region of the bulk 16S rRNA genes was amplified by PCR as described by Muyzer et al. [37]. The obtained PCR amplicons were separated by electrophoresis on a polyacrylamide gel with an increasing gradient of denaturants [37]. Following staining with SYBR® Gold (Invitrogen) and visualisation with UV illumination, the DGGE fingerprint gel was numerically analyzed using the BioNumerics 4.61 software. The DGGE fingerprints were clustered using UPGMA and relying on Pearson product-moment correlation coefficient.

2.4. Experimental design

Different sets of experiments (steps A–B–C) were performed on SS samples in different mesocosms (at the ISMAR Laboratories and at the Genoa Aquarium) and in an open-system (at the ISMAR Marine Station). The SS samples were tested in the mesocosms in open circuit conditions (step A); a test in potentiostatic conditions was furthermore performed in the mesocosm that showed the best results in the preliminary experiment in open circuit conditions (step B). Finally, a potentiostatic experiment was performed in the open-system (step C).

During all experiments, the measurement of the electrochemical activity of biofilms was associated with the study of percentage covering and genetic diversity of settled biofilms.

2.4.1. Step A: different volume mesocosms, same electrochemical technique (OCP)

In the OCP experiments the increase of potential value (measured with a Keithley 614 electrometer vs. Ag/AgCl electrode filled with KCl