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# Comparison of real time impedance monitoring of bacterial biofilm cultures in different experimental setups mimicking real field environments

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ABSTRACT

Bacterial biofilms are presented in many different environments causing a wide variety of infectious processes. Biofilms at their mature stage are difficult to eradicate because of their inherent resistance to antimicrobial agents. Easy-to-integrate and *in situ* detection tools would provide early detection of bacterial presence allowing efficient prophylactic actions. Impedance microbiology has been postulated as a suitable technique that allows monitoring of bacterial biofilm growths in real time. In this work four different culturing setups were developed as testing platforms for measuring real time microbiological cultures that could mimic real field environments. Results suggest that the position of the sensors in regard to the dynamic conditions of the culture might affect the sensitivity and the target parameter. Capacitance and resistance are associated to different biological effects, surface coating and conductivity changes respectively. Relative variations of electrical parameters were recorded in the lab obtaining significant changes in few hours post-infection. It has been proven that biological coating cause largest variations in capacitance, up to 60%, while metabolic activity affects more the resistance giving a variation up to 15%. Fitting analysis has confirmed experimental results showing also the effect of the dead/alive ratio.

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## 1. Introduction

As a consequence of the ubiquity of microorganisms, bacterial biofilms are a leading cause of severe problems in a wide variety of environments: healthcare, food industry, water and oil pipelines, etc. [1–3]. These biological structures present high resistance to antimicrobial treatments in their mature stage, leading in many occasions to chronic pathologies [4,5]. Early detection of bacterial presence could provide a better treatment scenario where the effectiveness of antibiotics would be increased.

Biofilm-related infections present high risks for the health of patients, especially for those who are immune compromised. Central venous catheters (CVCs) or central venous ports (CVPs) have changed medical care for treatments such as chemotherapy. However, these devices have an elevated risk of hosting biofilm colonization due to the significant consequences of exposure during device manipulation. Episodes of biofilm colonization often result in surgical intervention, prolonged hospitalization and high doses of antibiotic treatments. In addition to the negative impact on patient health, these procedures also result in an additional cost burden for the healthcare system [6,7]. Although the efforts of this work are focused on the development of IDE biosensors to be integrated in implanted devices, it is worthwhile to consider the diverse range of additional applications in a number of other problematic areas.

Impedance microbiology has been largely applied to microorganism detection in many different areas. The target sample is placed on a device equipped with electrodes allowing an impedance measurement. Electrical properties of these biological systems are one of the simplest but more effective ways to identify their presence. In this context there are different methodologies that could be applied for the detection using different elements as the recognition target [8]. Some of the works that use impedance based methods for bacterial detection combine the electrical measurements with other binding techniques. Antibodies are the most common biorecognition elements, typically immobilized on the sensing surfaces [9], magnetic beads [10], and the dielectrophoresis effect [11] have also been successfully utilized for specific detection.

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Previous works have demonstrated the effectiveness of label-free impedance spectroscopy analysis for biofilm development monitoring in *in vitro* testing with different culturing setups [12,13]. Both long term monitoring and rapid sample analysis [14,15] show that the low frequency range is ideal to directly detect the presence of microorganisms.

Continuous *in situ* or *in vivo* monitoring of the target fluid will allow bacterial detection at the first stages of microbe development. However the detection method will require monitoring the growth over a time interval to rule out other transitory or non-steady-state effects. Unfortunately, each one of the variables of the environment will also affect the bacterial growth kinetics. For that reason it is necessary to develop new *in vitro* setups for more realistic characterization of the *in situ* growing conditions.

Therefore the main goal of this work is the comprehension and comparison of the behavior of bacterial cultures under different growing conditions by the analysis of impedance response of *in vitro* cultures. Four different culturing setups are presented as culturing platforms and impedance monitoring. Impedance variations are analyzed for each setup regarding the specific culturing conditions as well as the fitted values from the electrical model proposed. Experimental and simulated data are compared, extracting conclusions on the dead/alive ratio of bacterial cells and its implication on the impedimetric response.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Tryptic Soy Broth, TSB (BBL™, ref: 211768) enriched with 5% glucose (Dextrose from Difco™, ref: 215530) was used as culturing media. Phosphate buffered saline, PBS (0.01 M, pH 7.4) purchased from Sigma–Aldrich (ref: P5368-10PAK), saline 4.5% solution from Panreac (PA-ACS-ISO ref: 131659) and Brain Heart Infusion, BHI (Bacto™, ref: 237500) were also used at different steps. All solutions and media were prepared with deionized water (Merck Millipore). Media was sterilized at 121 °C for 1 h in the autoclave.

Ethanol at 99.5% (Panreac ref: 131659) and 10% Hellmanex II dissolution (Hellma Analytics ref: 9-307-010-507) were used for cleaning biosensors.

### 2.2. Microorganisms and culture protocol

A bacterial strain of *Staphylococcus epidermidis* (SE) ATCC 35984 was used in this study. It belongs to the American Type Culture Collection (ATCC) and was purchased from the Colección Española de Cultivos Tipo (CECT). This strain was selected due to its high ability of forming biofilms. Before its utilization, the strain was stored frozen at −80 °C in a Cryoinstant® mixed vial (pH 7, 2 ± 0, 2, obtained from Scharlau ref: 064-TA8276).

Sample preparation was performed following the different steps described in a previous manuscript [12]. A 0.5 McFarland suspension was prepared with the cryogenic samples following the standard proceedings of CLSI (Clinical and Laboratory Standard Institute, 2009). Bacterial concentrations were performed following the standard plating methods. The number of microorganisms present in each sample was determined by plating 100 μL onto a culture plate.

### 2.3. Experimental setup and biosensor

Four different experimental setups were designed and fabricated *ad hoc* for bacterial monitoring assays based on different traditional culturing methods or clinical devices: 96-well microtiter plates (WMP), petri dishes (PD), modified CDC biofilm reactor system (MCDC) and a lab-tester (LT) device that simulates a central venous port (CVP). The modified CDC biofilm reactor system and the 96-well array plate's setups were reported in previous works, [13] and [12] respectively. Fig. 1 shows both CAD images and real field photos of the four devices during experimentation.

MWP and PD (Fig. 1(a) and (b)) use the same chip holder and sensors on different culturing plates. Eight sensors are hold vertically and submerged in the wells or in the dish in each case. A connection board allows electrical connection of each sensor to the measurement equipment. The modified CDC biofilm reactor (Fig. 1(c)) is based on a 1 L glass and six chip holders with 3 sensors of capacity each one. Sensors are submerged in the culturing media and could be removed at any time during the experiment. Inlets and outlets as well as stirring allow changing the dynamic conditions of the culture. Shear stress caused by the stirring effect will favor the biofilm proliferation attached to the free surfaces of the vessel. Each chip holder is hermetically sealed so the liquid could not interfere with the electrical connections. Finally, the lab-tester device (Fig. 1(d)) consists of a main body with a reservoir chamber sealed with a silicon cap, an outlet and a sensor located and sealed at the bottom of the reservoir. The lumen of the device is accessible through the silicon cup with a needle allowing the modification of the dynamic conditions of the culture during its performance.

Table 1 summarizes the most relevant features of the setups: volume, shaking and flow dynamics, which relate the accuracy of the models compared to real situations. In addition, the behavior of the culture could be easily modified by adjusting the dynamic conditions of each culture vessel by adding stirring/mixing, providing flow, or combining stationary and transient phases.

All setups allow sterilization before experimentation by autoclaving the system for 1 h without any structural or functional damage. The main components of these four setups were designed to be reusable so the chip holders provide a mechanism to lodge and substitute sensors and connections for each experiment. However, there are some problems related to culturing conditions inside the incubator when working with small volumes. For the WMP setup, it has been reported that after 50 h of culture, the decreasing level of the media begins to affect impedimetric measurements. Improved plate sealing has been suggested as one potential solution for this problem.

Each one of the four setups provides a different environment for bacterial biofilm growth; this allows analysis of different growing effects. The WMP and the PD could be useful for mimicking food industrial models of bacterial contamination, such as contamination of packaged food, small volumes of stagnant water or liquids. The MCDC can be utilized for the same purpose for industrial device models, including heat exchangers, water treatment plants, and water or fuel pipelines for example. Finally, the LT is appropriate for imitating the performance of medical devices such as CVPs, CVCs, and dialysis lines. The same type of silicon sealing was used on the LT devices during experimentation. Eventually all these setups could be useful for treatment testing using impedance microbiology to track chemical efficacy [16].

The holder used in WMP and PD as well as the one used in the MCDC also could be placed in bigger vessels or even in *in situ* or real field samples. This possibility potentially allows comparison of the *in vitro* results with real field measurements.

### 2.4. Biosensors and impedance measurements

The sensors used for impedance spectroscopy measurements were designed and fabricated by means of silicon microtechnology on 3 in. wafers. The IDE biosensors consist of a 100 nm thin film layer of gold sputtered onto a 15 nm chromium adhesion layer deposited onto the thermally oxidized silicon wafer.

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