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ORIGINAL PAPER

The Application of Impedance Microsensors for Real-Time Analysis of *Pseudomonas aeruginosa* Biofilm Formation

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Abstract

Biofilms formed by nosocomial pathogens represent a major threat to patients undergoing invasive procedures. As prophylaxis remains the most efficient anti-biofilm option, it is of paramount importance to develop diagnostic tools able to detect biofilm at the early stage of formation. The present study investigates the ability of impedance microsensors to detect *Pseudomonas aeruginosa* biofilm presence using the impedance spectroscopy method. The measured data were analyzed using Electrical Equivalent Circuit modelling (EEC). It allowed to recognize conduction and polarization phenomena on the sensors surface and in its environment. The impedance assay results, confirmed by means of electron microscopy and quantitative cultures, indicate that specific EEC parameters may be used for monitoring the development of pseudomonal biofilm.

Key words: *Pseudomonas aeruginosa*, biofilm, impedance sensors

Introduction

The majority of bacteria exist in settled communities, referred to as biofilms. Contrary to their planktonic (free-swimming) counterparts, bacterial cells in biofilm are embedded within an extracellular matrix, which serves the bacteria as a shelter and a shield protecting from antimicrobials and immune system components. Biofilms develop easily on tissues (most preferably damaged) and on abiotic surfaces (*i.e.* dressings, catheters, intubation tubes (Flemming *et al*., 2008; Pradeep *et al*., 2013; Bjarnsholt *et al*., 2008).

Biofilms are responsible for up to 80% of nosocomial infections (James *et al*., 2008). Presently, there are neither biofilm-resistant medical implants nor procedures guaranteeing biofilm eradication. The only exception, to some extent, are non-specific actions such as surgical removal of infected tissue or removal of a colonized implant. However, the above mentioned procedures are not always possible or safe for the patient.

Therefore, there is an urgent need to develop diagnostic tools for an early detection of biofilm presence. Sensors, measuring physical value referred to as impedance, display promising properties that may be used for this purpose (Ben-Yoav *et al*., 2013; Zhenga *et al*., 2013; Padresa *et al*., 2013). Impedance describes the potential of natural objects to resist the electric current flow and to store energy in the form of electric or magnetic field. All physicochemical changes occurring within biological systems have an impact on their electrical properties and affect the impedance value. Also such important biological phenomena as fluctuation of ionic concentration, cell division or adhesion to the surface, may be potentially detected by impedance sensors (Ge *et al*., 2008; Hakki and Bozkurt, 2011). The most important advantage of using impedance microsensors is the possibility of real-time monitoring of these changes. Other advantages are non-invasiveness, label-free detection (no markers needed) and provision of data concerning electrical properties of the environment.

The detection of bacteria by means of impedance microsensors may be helpful in virtually all flow systems endangered by the development of microbes. Among the examples of such systems in nosocomial

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settings are indwelling catheters, nutrition accesses or hospital water distribution pipes (Taeyoung *et al*., 2011; Munoz *et al*., 2006).

As the majority of impedance-related research on biofilm is still at the preliminary level (Dominguez-Benetton *et al*., 2012), the aim of this study was to contribute to the exploration of this issue. Thus, specially designed impedance microsensors were applied for the real-time analysis of biofilm growth of *Pseudomonas aeruginosa*, a recognized nosocomial pathogen.

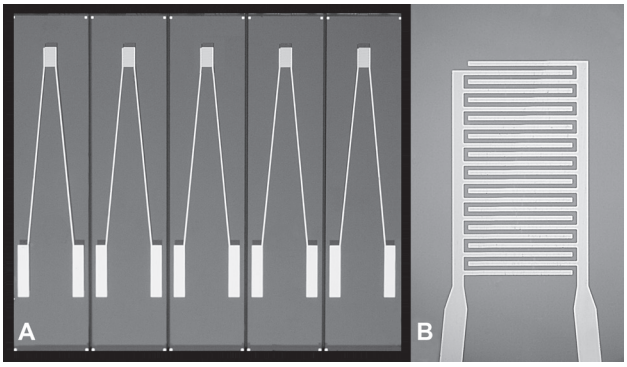


Fig. 1. Left side: Impedance microsensors on a glass substrate
Right side: IDE structure of sensor. Digits of electrodes are visible

Experimental

Materials and Methods

Impedance microsenors with interdigitated electrodes (IDE) were used in the experiment. The electrodes were made of vacuum-evaporated gold on PYREX glass with a titanium adhesion layer. The distance between the electrodes and the width of the electrode digits were 20 μm. The area of the electrodes was 0.6×1 mm. The shape and contact pads placement of the sensors allowed to mount them manually to the micro USB plug and to place them vertically in the wells of a 24-well plate (CellStar, Germany). The sensors used for the experiment are presented in Fig. 1. To prepare electrodes, sensors were rinsed in distilled water, then in acetone and in isopropanol, finally.

GW InstekLCR-8101G was used as Impedance Analyser (IA). The measurement system was designed to handle 8 sensors. It consisted of a suitable 8-channeled switch and the above-mentioned IA (Fig. 2).

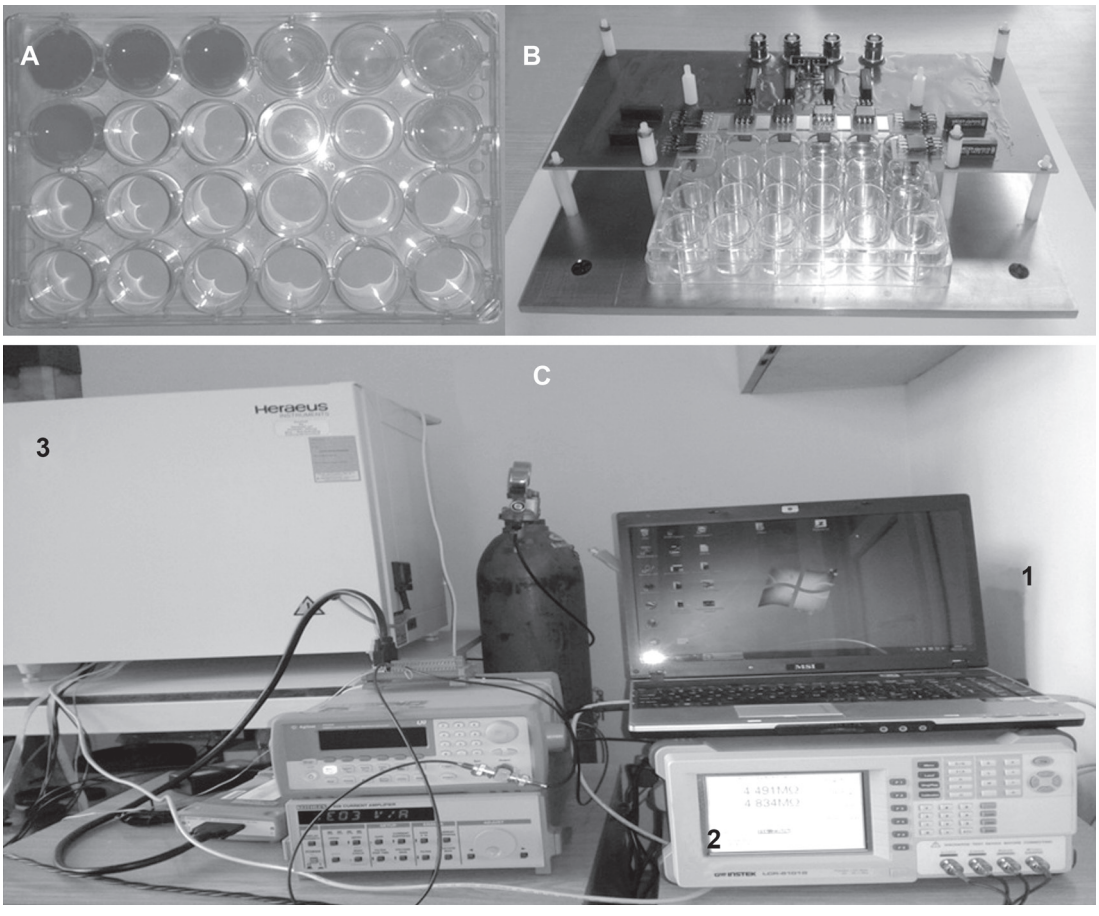


Fig. 2. Upper left side: A 24-well plate filled with *P. aeruginosa* culture (left side) and a sterile medium (right side)
Upper right side: 8-channel switch with sensors dipped in the wells. After connecting to the impedance analyser, the switch is placed in the incubator.
Bottom. Work station for impedance biofilm assays: 1 – PC with ImpeDancer software; 2 – Impedance Analyser; 3 – incubator of 24-well plates

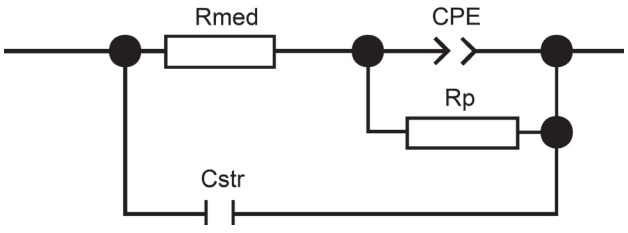


Fig. 3. Electrical equivalent circuit (EEC) of the impedance sensor in bacteria-free and bacteria-containing medium

Particular parts of the EEC represent the following electrical properties of measured object: Rmed – medium resistance, Cstr – sensor's structure parasitic capacitance, CPE – capacitance of electrodes-environment interface, Rp – parallel resistance to CPE

Such a setting allows to perform quasi-simultaneous measurement of the sensors placed in different wells of a microtitre plate. The measurement system was controlled by a PC computer with a customized software described in more detailed manner in (Piasecki *et al.*, 2012) (Fig. 2).

Real-time impedance measurement of biofilm formation. A reference *P. aeruginosa* ATCC 14454 was used for experimental purposes. The ability of the aforementioned strain to form biofilm on abiotic surfaces has been already recognized in the previous work of the authors (Junka *et al.*, 2013). An overnight culture of the investigated strain was diluted to 1 McFarland using a densitometer (Biomerieux, Poland) and subsequently to 10^3 cfu/ml using dilution method. Two ml of the strains' culture was introduced to the wells of the plate. Next, the sensors were mounted aseptically in a 8-channel switch and placed in the wells. Impedance spectra of each sensor were measured for 24 hours/37°C using the impedance spectroscopy method with four minute intervals. Pure Tryptic Soya Broth (TSB, BioMerck, Poland) was used as a negative control of the experiment.

The obtained impedance spectra were analysed using the Electrical Equivalent Circuit (EEC) method. A special EEC was created for this experiment (Fig. 3). It allowed to model conductivity and polarization processes occurring in the impedance sensor area (medium and biofilm). Two EEC components describe the phenomena taking place on the electrodes of the sensors. These are CPE and Rp (capacitance of electrodes-environment interface and parallel to it resistance, respectively). The admittance of CPE is given by $Y_{CPE}(\ )$ where Q and n are the parameters and ω is a radial frequency (Barsoukov and Macdonald, 2005). The values of these parameters were calculated for each of the time-points using ZView software by Scribner. Rp, Q and n were analysed with regard to the process of *P. aeruginosa* biofilm formation.

Quantitative cultures. The *P. aeruginosa* strain and the impedance apparatus were prepared as described above. The strain was incubated in the presence of sensors for 2, 6, 12 and 24 hours. Next, the sensors were aseptically removed from the apparatus, rinsed with saline, and transferred to 1ml of mild detergent – 0.5% saponine (Sigma Aldrich, Poland). The sensors were vortex mixed for 1 minute to remove biofilm from their surface. Subsequently, the obtained suspensions were diluted 10–10^7 times. 100µl of each dilution was cultured on a McConkey Agar Medium (Merck, Poland) and incubated at 37°C for 24 hours. Next, the bacterial colonies were counted and the number of bacterial cells forming biofilm was assessed.

Electron microscopy. The strains were allowed to form biofilm on the sensors' surface under the conditions described above. Subsequently, the sensors were aseptically removed from the apparatus, rinsed 3 times with saline to remove non-adherent bacteria and dried at 37°C/4 hours. The dried samples were covered with Au/Pd (60:40, sputter current: 40 mA, sputter time: 50 sec) using QUORUM machine (Quorum International Forth Worth, USA) and examined on Scanning Electron Microscope Zeiss EVO MA25.

Results

Biofilm dynamics. After two hours of incubation, pseudomonal cell clusters were found on the sensors' surface (Fig. 4, upper left side). Quantitative cultures revealed the presence of *ca* 10^4 colony forming units of bacteria on the sensor. During the next four hours, the number of bacteria increased to 10^8. Therefore, this period may be identified as an intensive growth phase. The surface of the sensors was partially covered with a dense multi-layered bacterial biofilm. Although slower than previously, after 12 hours from the beginning of the experiment the number of cells was still increasing. This suggests the phase of biofilm maturation. After 24 hours, virtually the entire surface of the sensor was covered with biofilm in different stadia of development (Fig. 4, lower right side).

Electrical measurements. Changes of the environment's physicochemical properties detected by the sensors are presented as a set of impedance spectra in the form of serial capacitance and dissipation factor (Fig. 5). The measured data were analysed using Electrical Equivalent Circuit (EEC) modelling.

Such approach allows to obtain qualitative information about the measured object from impedance spectra. As can be seen in Figure 6, all three analysed parameters – Rp, Q and n, were informative for the purposes of analysing *P. aeruginosa* biofilm formation. The Q parameter was found to be positively correlated with increasing number of cells on the sensors, whereas the n value decreased along with biofilm development (Fig. 6A-B). From the 6th hour of the experiment, the

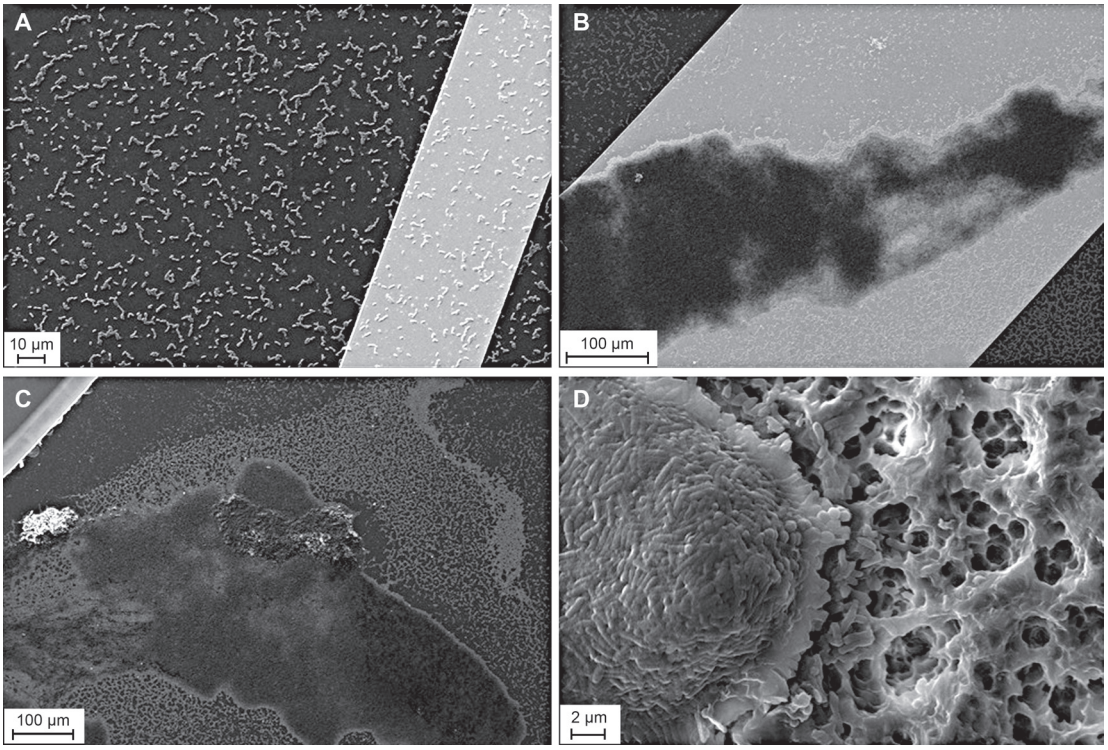


Fig. 4. Upper left side: Pseudomonal cells on the surface of impedance sensor after 2 hours of incubation Magn.x1450. Upper right side: Pseudomonal cells on the surface of impedance sensor after 6 hours of incubation. Different stadia of biofilm formation are visible. Magn.x441.Lower left side: Pseudomonal cells on the surface of impedance sensor after 12 hours of incubation. Different stadia of biofilm formation are visible. Magn.x325. Fig Lower right side: Multi-layer pseudomonal biofilm on the surface of impedance sensor after 24 hours of incubation. Both the extracellular matrix and pseudomonal cells are visible. Magn.x9070. All pictures presented were taken using Electron Microscope Zeiss Evo MA 25

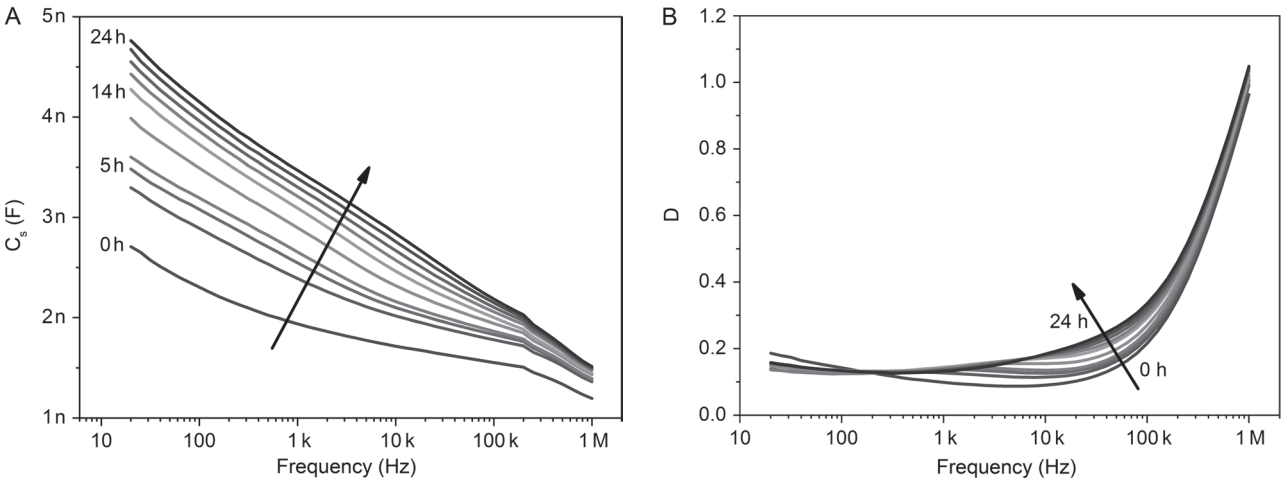
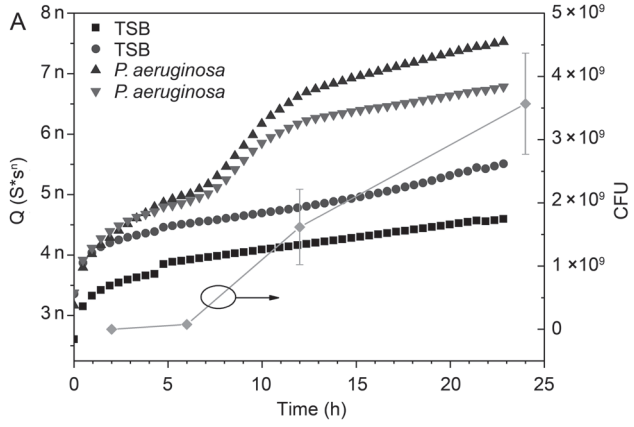


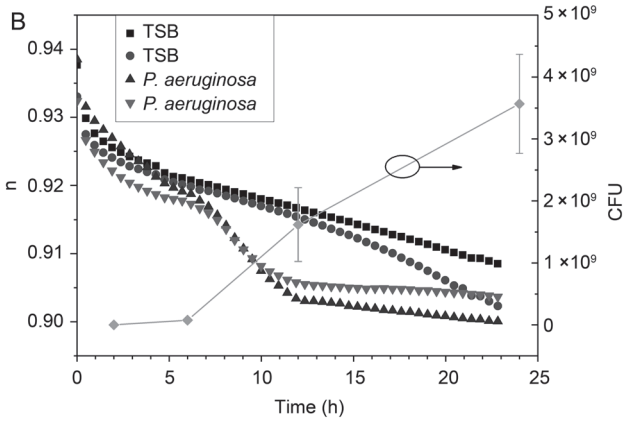
Fig. 5. Typical spectra of serial capacitance C_s and dissipation factor D in time registered in *P. aeruginosa* filled wells

differences in Q measured in the control and tested well started to be visible and such a state continued for another 5 hours. Afterwards, the value of the Q parameter in the tested sample resembled that of the control again. A different type of results was obtained for Rp. The value of this component was slowly and uniformly increasing until the 11-th hour of the experiment and increased rapidly afterwards, reaching the value of 10^{15} ohm (Fig. 6C).

Discussion

In the *in vitro* setting applied, pseudomonal biofilm underwent all specific stages of development, including adhesion, matrix synthesis and maturation (Fig. 4). As it was shown, the impedance sensors used were able to distinguish between bacteria-free and bacteria-containing environment. This ability offers application potential, especially as the value of one of the param-





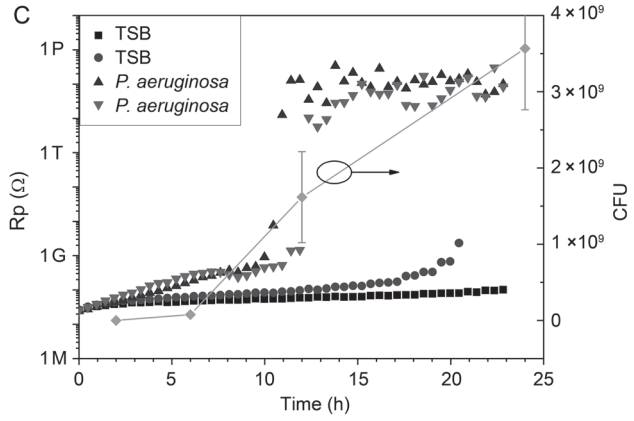


Fig. 6. Upper left side: Values of the Q parameter of EEC component CPE, correlated with changes of *P. aeruginosa* cfu number Each plot represents different series of measurement. The ellipse with the arrow points out right scale of the graph describing values of cfu. Upper right side Values of the n parameter of EEC component CPE, correlated with changes of *P. aeruginosa* cfu number. Each plot represents different series of measurement. The ellipse with the arrow points out right scale of the graph describing values of cfu. Bottom: Values of EEC component Rp correlated with changes of *P. aeruginosa* cfu number. Each plot represents different series of measurement. The ellipse with the arrow points out right scale of the graph describing values of cfu

eters analysed, namely Q, started to rise as early as after 2 hours from the start of the experiment, along with an increasing bacterial cell number. Moreover, Q values seemed to correlate with the phases of bacterial growth on the surface of the sensor (Fig. 6A).

On the other hand, during the first 6 hours, the differences of the n value measured for control and bacteria-containing environment were not as distinct as in the case of the Q parameter. Thus, it may limit the potential applicability of the n parameter for biofilm detection. Further experiments should be performed to interpret the results obtained for n, with special attention to the phenomena taking place between 6^{th}–11^{th} hour from the beginning of the experiment.

Puzzling results were obtained for Rp, the third parameter analysed. After the 11^{th} hour of incubation, the value of this component increased significantly. According to the electrical model applied, such a phenomenon may be explained by a loss of DC conductivity. It might be the result of biofilm development on the entire surface of the electrodes. However, the discussed rise of the Rp value took place late in the course of the experiment. At that time, vast biofilm clusters formed on the sensors were seen not only by means of electron microscopy (Fig. 4) but also with the naked eye. Therefore, Rp for biofilm detection is useful only in systems where optical detection is impossible.

Still, at least one of three tested parameters, namely Q, may be useful for the analysis of bacterial biofilm formed on the sensors' surface. This finding, confirmed by electron microscopy and microbiological techniques, may be helpful in future development of clinical applications. Biofilm-related infections represent a major threat to patients undergoing invasive nosocomial procedures. The presented study aims to be another brick in the wall built for the patients' protection.

Summary

- The applied impedance microsensors are able to detect bacterial presence
- Electrical Equivalent Circuit modelling is useful for analysing sensor response
- The applicability of Rp and n parameters for the detection of biofilm presence seems to be limited.
- The value of Q changes along with biofilm phase of growth. The suitability of this parameter for biofilm detection is promising, although further experiments need to be performed.

Acknowledgments

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