MEASUREMENT OF BIOFILM THICKNESS: AN EFFECTIVE LEGIONELLA RISK ASSESSMENT TOOL

CHRISTOPHE FORET, NICOLE MERLET, GUÉNOLÉ CHAUSSEC, SERGUEI MARTEMIANOV, BERNARD TRIBOLET AND WOLFGANG HATER

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ABSTRACT
The best way to prevent the risk of bacterial growth in water systems is to monitor and to control the microorganisms (biofilm) attached to pipe walls. Three years of laboratory research led 2 CNRS teams (UMR 6008 and UPR 15) to develop a tool designed to determine the average biofilm thickness. The average biofilm thickness measurements carried out on pilot plants fed with natural water were sufficiently accurate and sensitive to monitor the formation and development of biofilm in a water system and to determine the efficiency of the applied treatments. The implementation of appropriate treatments (type and dose of the treatment product) leads to a significant reduction or even complete removal of the porous layer on the material surface. The reduction of the attached biomass, measured by the sensor, is connected to the decrease of the bacterial density attached to the material (viable flora in PCA environment).

INTRODUCTION
The growth of biofilm on pipe walls may cause serious sanitary problems (growth of Legionella pneumophila or Pseudomonas aeruginosa bacteria, or the Naegleria fowleri ameba) in water systems (hot sanitary water, drinking water, cooling towers, cooling systems). As regards Legionnaire’s disease (also known as legionellosis), the number of cases reported in France has progressively increased from 1044 cases in 2003 to 1201 cases in 2004, and reached 1527 cases in 2005, despite the implementation of additional guidelines relating to the monitoring and prevention of legionellosis in 1997; the fatality rate is approximately 14%. The “Plan National Santé Environnement” (PNSE) (National Health and Environment Plan), adopted in 2004, set the target of reducing by 50% the number of legionnaire’s disease cases by 2008, and the enforcement of more stringent rules and regulations (Order 2004-1331 dated 1st December 2004) in order to limit the effects of legionella bacteria.

In 2004, the French Government drew up an inventory of more than 12,000 cooling towers installed on approximately 6,000 buildings just for industrial plants and service industry buildings alone. Concerning risk prevention in these cooling towers, they require regular maintenance and upkeep, the monitoring of facilities, the implementation of preventive and curative procedures, and the optimisation of cleaning and disinfection techniques. Despite these efforts, bacterial contamination cases still regularly occur in facilities, the exact causes of which are not known, and there are no fast effective means to identify and prevent them.

In this context, therefore it would appear essential to install a sensor in order to monitor the development of the biofilm and to design an effective tool to help the operators of hazardous facilities to take rapid action. Several research programs aiming to develop such a tool have been conducted during the last ten years. These programs led to the design of sensors, such as the BioGeorge™ system (Brujs et al., 2001) or the BIOX sensor (Mollica, 2000), and more recently, the Biomosys™ microsensors (Desmarest, 2006), but we still lack feedback on such systems, and technological issues still have to be solved for some of them.

Therefore, we set out to develop an electrochemical detection system to monitor the transport of matter through the porous layer formed by the biofilm. Based on the research of Herbert-Guillou et al., decided to focus our research on a rotating disc electrode measurement system, easier to use and enabled us to collect more information.

A SIMPLE ELECTROCHEMICAL SYSTEM
A porous layer (e.g. biofilm), covering the surface of a metallic electrode, acts as a diffusion barrier.

The thickness of attached biomass is measured with a rotating disc electrode device, which, immersed in an electrolyte, constitutes the electrochemical system which can be used to analyse the transport of matter. Thanks to a rotating movement, this system sucks in the liquid and conveys it to the electrode, at the surface of which a hydrodynamic boundary layer is formed. The signal studied is the current corresponding to an electrochemical reaction entirely limited by the transport of matter, and occurring at the metal/biofilm interface. When there is a porous layer, the
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transport of matter and therefore the current are reduced, which means the thickness of this porous layer can be measured.

To be more precise, the aim is to analyse the difference between the current measured when there is biofilm and the current measured when there is no biofilm. For this measurement, we consider that the diffusion coefficient in the biofilm is similar to the diffusion coefficient in the electrolyte, as the biofilm contains over 95% water (Flemming et al., 1999).

The test measuring cell is made up of three electrodes immersed in an electrolyte containing the electrochemical tracer: work electrode (rotating disc electrode), counter electrode and saturated calomel reference electrode (SCE) (figure 1).

The work electrode (rotating disc electrode) has a detachable tip used as a sensor to measure the biofilm thickness. The electrodes used in the laboratory are those usually sold on rotating disc systems; we chose an active surface with a diameter of 2 to 5 mm in platinum. For industrial systems, this is a platinum cylinder, whose side surface is embedded in an insulating material.

Finally, a data acquisition device is added to this cell. It has a potentiostat, which may be associated with a computer for data acquisition purposes.

Before the sensors are introduced into the measurement environment, the platinum surface of each measuring electrode is thoroughly cleaned with sandpaper (silicon carbide: 4,000 grain size), and then rinsed with distilled water. The current measured for a clean surface will then correspond to a biofilm zero-thickness.

**ACCURATE, RELIABLE AND REPRODUCIBLE MEASUREMENTS**

Tests carried out on pilot plants for over 3 years have shown the capacity of this tool to determine accurately the thickness of biofilms of various origins.

The incubators used (figure 2) are made up of glass columns (length = 50 cm; diameter = 3 cm) in which measuring electrodes are carefully inserted with their reactive surfaces level with the internal surface of the column. Incubators are permanently fed with water of varied origin (e.g. river water, dam water, underground water), and the desired flow rate is ensured by recirculation.

An additional incubator with glass supports (balls and/or blades) may be added to this testing assembly in order to quantify the biomass attached to the support (after detachment by sonication), or to examine them using microscopy techniques.
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**Figure 2: Diagram of the tubular incubator**

Figure 3 represents voltammetric measurements [\( I=f(E) \)] during the development of a water river biofilm within a 15 day period. The value of the limiting current, a parameter measured at the diffusion plateau (for a potential ranging from -0.3 to +0.2 V/SCE), decreases when immersion times increase, thus with the growth of the biofilm. The reduction of matter transport at the surface of the electrode characterises the development of biofilm, in particular the increase of its thickness.

Average thicknesses measured for different types of water vary significantly according to the biofilms and may reach a few micrometres (figure 4) for an immersion time of 10 days. Measurements read on 4 sensors placed on the same pipe show that this measurement is accurate, reliable and reproducible (figure 5), and that the heterogeneous distribution of the biofilm on a support, true on a microscopic scale, is not sensitive on a macroscopic scale (surface of the electrode used as a sensor). This method determines precisely average thicknesses greater than or equal to 1 µm.
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Figure 4: Thickness of some biofilms after 10 days of colonisation (electrode rotating speed = 300 rpm)

Figure 5: Evolution of the average biofilm thickness during its formation and development (measurements carried out on 4 sensors placed in the same pipe)

Sagittal sections (profiles according to vertical planes) in confocal microscopy on some of these biofilms also enabled us to control these results by assessing the maximum thickness of deposits (figure 6). Thickness may vary significantly according to the location in the deposits, and the maximum values recorded for these biofilms formed in 16 days range from 10 to 20 µm.

This microscopy technique also shows how these deposits are organised. On figure 6, colouring with fluorescent probes (lectin-TRITC, SYTO9) and imaging obtained on 3 channels enabled us to locate bacterial cells, appearing in green, exopolymers in red, and the photosynthetic biomass in blue. It shows that the spatial distribution of the biological and organic fractions of biofilms varies significantly between the bottom and the surface of the biofilm. Microscopy examinations carried...
out on several biofilms show that the bacterial biomass (coloured in green) represents a proportion generally lower in the upper part of the deposits.

However, this technique does not highlight the mineral matrix of biofilms, which may represent an important component of these biofilms.

**Figure 6: Sagittal sections in confocal microscopy of 2 biofilms formed in 10 days with surface waters**

**RELATION BETWEEN THE MEASURED AVERAGE THICKNESS AND THE BIOFILM MATRIX**

According to the nature and characteristics of the circulating water, the composition of biofilms varies significantly from one to another. Biofilms are mainly composed (in terms of mass) of organic (extracellular polymeric substances in particular) and mineral matrices, but in variable proportions, the bacterial flora is only representing a minor component.

Therefore, some biofilms are mainly made up of organic matter, whereas others are mainly composed of mineral compounds, such as calcite (CaCO₃), or Fe, Al, Mn (Hiernaux, 2005) hydroxides or oxo-hydroxides. Given this variability of composition, it is therefore not surprising to find no direct connection between the biofilm average thickness and its exopolymer content (figure 7). However, data on this last figure clearly show that the smallest thicknesses (< 3 µm) are associated with low-organic carbon biofilms (< 600 ng/cm²).

**Figure 7: Organic matter contents (expressed in organic carbon) and thicknesses of some natural water biofilms (age = 10 days)**
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As regards the bacterial risk, assessed by determining the *Legionella pneumophila* content in the biofilm matrix (AFNOR culture method in BCYE environment, after biofilm detachment by sonication), the same findings can be put forward (figure 8). The biofilm thickness is not directly related to the *Legionella* content. However, the figure clearly shows that waters with biofilms which did not permit the growth of *Legionella* (BP wells, Flée source, Viette and Vézère rivers) have low average thicknesses (3 µm).

![Figure 8: Legionella pneumophila contents and thicknesses of natural water biofilms (age = 10 days)](image)

**TOOL APPLICATION TO THE MONITORING OF TREATMENT EFFICIENCY**

The capacity of this sensor to assess the efficiency of dispersing or disinfection treatments on pipes colonised by biofilms has been tested during research on the above mentioned pilot plants. The application of different dispersing molecules at constant doses in pipes previously colonised for 10 days at 37°C with surface waters enabled us to monitor and to compare the efficiency of these molecules in real conditions (figure 9). On a biofilm with an initial thickness of almost 13 µm, dispersing agents A and B began to detach the biofilm only after a 3 hour contact time, whereas products C and D disintegrated the biofilm matrix more quickly. However, all products are equally efficient with a reduction of 60-70% of the average biofilm thickness after 5 hours of treatment.

![Figure 9: Impact of treatment with dispersing reagents on the biofilm thickness](image)
The impact of treatment products, measured thanks to the biofilm sensor, was correlated with the reduction of the bacterial density attached to the material (figure 9). Applied at a treatment rate of 10 mg/L, the dispersing product E reduced the biofilm thickness from 6.4 µm to 2 µm after a 5 hour contact time, whereas increasing the dose to 50 mg/L fully removed the porous layer (zero-thickness) (figure 10). In the same way, the measurement of the attached bacterial density (figure 10 b) carried out after 5 hours of treatment shows a significant effectiveness at a dose of 10 mg/L, with a bacterial reduction of 2 log (number of bacteria divided by 100), but $10^3$ UFC/cm² still remain. On the other hand, with a higher treatment dose (50 mg/L), the material surface is almost bacteria-free with a reduction greater than 4 log (initial number of bacteria divided by 10,000).

Figure 10: Influence of the treatment with dispersing products on:

a) biofilm thickness - b) viable bacteria density in the biofilm
CONCLUSION
The electrochemical sensor developed as part of this research is an accurate, sensitive and reproducible analytical tool for measuring the transfer of matter within the porous layer made up by the biofilm on the surface of a material. The sensor measures the average thickness of this biofilm, and the measurement is not destructive, thus allowing the monitoring with the same sensor over the course of time. The minimum thickness that can be measured is estimated at 0.5 µm, and the biofilms, developed in a few days at 37°C on pilot plants, have thicknesses ranging from 3 to 15 µm, whereas the measured thicknesses with underground waters reach a maximum of 2 to 3 µm after ten days.

Research in the laboratory and on pilot plants has shown the capacity of this sensor to assess accurately the effectiveness of the procedures used to monitor and control the biofilm. In fact, the analytical tool is sufficiently accurate and sensitive to:

- assess the impact of a treatment (cleaning, disinfection)
- distinguish the different treatment products,
- provide an answer in correlation with the density of the attached bacteria,
- highlight any resistance to treatments.

The development of an industrial sensor inspired by this research is underway with an industrial partner (BK Giulini).

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CONTACT

Christophe Foret
BKG France SAS
43, Rout de Ruaudin
72230 Arnage
France
E-mail: Christophe.foret@bk-giulini.fr

Wolfgang Hater
BK Giulini GmbH
Niederheider Straße 22
40589 Dusseldorf
Germany
E-Mail: wolfgang.hater@bk-giulini.com

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BKG Water Solutions
Headquarters:
BK Giulini GmbH
Giulinistrasse 2
D - 67065 Ludwigshafen
Phone: +49-621-5709-01
Fax: +49-621-5709-452

Office Dusseldorf:
BK Giulini GmbH
Niederheider Str. 22/Geb. Y20
D - 40589 Dusseldorf
Phone: +49-211-797-9190
Fax: +49-211-798-2262

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