WRAPPED BY BACTERIA – BIOFILM DETECTION AND REMOVAL IN PAPER MILLS

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Abstract: Biofilm growth inside paper mill water lines is a major problem, since bacteria can alter the quality of paper and damage machinery. To fight them, large amount of chemical substances are usually applied, without a link to the real biofilm development. Thus, there is a strong need for a monitoring technology able to provide an early warning, to optimize cleaning treatments. Several biofilm monitoring approaches have been proposed over the years, but their application has been very limited. Recently, a new technology able to measure biofilm electrochemical activity was successfully employed in different fields. In this work, the performances of this sensor for freshwater applications and, in particular, for paper mills have been evaluated both in lab and in a real case. The results show that, coupling standard biocide treatments with advanced on-line monitoring technologies, it is possible to keep track of chemicals and of their effectiveness toward biofilm.

Keywords: biofilm, sensor, paper mills.

Introduction

Microorganisms generally do not live in single isolated cells, but they rather tend to form organized communities (e.g. films, biological flocculates and sludge) that grow and develop, colonizing any available natural or man-made surface. Therefore, the term biofilm is generally used for all these types of microbial associations, irrespective of their biological structure and of the (natural or artificial) environment where they develop. Such an organic matrix consists of poorly water-soluble biopolymers, where organisms live and duplicate, competing with and replacing each other over time, until they form a dynamically balanced biological layer that covers the surface [1]. In freshwater environments, biofilm development is usually slower than in seawater, but its dynamics are similar.

When biofilm grows on any man-made equipment, it can cause serious wide-ranging technological problems, with strong negative economic effects. Bacterial proliferation inside the water lines of paper mills and, even more, biofilm growth on pipe walls, represents a major problem for the pulp and paper industry. Indeed, microorganisms can alter the quality of paper, causing modifications in the color and even lead to paper breaks. Machinery, too, can be damaged by this biological growth, including the risk of Microbiologically Influenced Corrosion (MIC) [2]. To fight these little but highly harmful organisms, large amounts of chemical biocides are routinely employed to treat paper mill process water [3]. Often, the cleaning treatment strategy is arbitrarily defined, without any information about the real biofilm development inside piping. Even in the paper mills that carry out a biological monitoring activity, the analyses are usually conducted on water samples taken from production lines, and rely on laboratory culturing assays (i.e. counting the number of colonies that develop on laboratory growth media, from a sample of process water). The first problem, intrinsic to this approach, is that the microorganisms growing on these kinds of media can be less than 1% of the total bacteria present in a water sample [4]. The second problem is that the number of free-floating bacteria (planktonic cells) present in the water is not directly correlated to the number of cells (sessile / biofilms) growing on the internal surface of piping[5]. Indeed, independently of the number of planktonic bacteria, once the first microorganisms from the water colonize a surface, they start to duplicate giving rise to the first thin layer of bacterial biofilm.

Biofilms represent the most difficult challenge for water treatment managers, since the resistance to

antimicrobial substances of bacteria growing in this form can increase up to 100-1000 times with respect to planktonic ones. This is mainly due to the production of extracellular polymeric substances (EPS) that shield sessile bacterial cells from external agents, including chemical biocides [5-6]. EPS are made of a whole set of different classes of macromolecules (polysaccharides, proteins, nucleic acids, and phospholipids) found in intracellular spaces of microbial aggregates. These molecules are responsible for cohesion forces allowing the biofilm matrix to acquire its typical three-dimensional architecture where microorganisms develop. Microbial influenced corrosion is further enhanced by EPS, which reduce the exchange of chemical compounds between water and material surfaces, thus altering the mechanisms and dynamics of bio-corrosion and biodegradation on the underlying surface.

With this perspective, the need is evident for an on-line, real-time, biofilm monitoring technology, able to provide early-warning indications on biofilm development to the technical staff in charge of water treatment. Such information would be extremely useful to optimize biocide treatments, making possible a prompt intervention as soon as biofilm appears, entailing a reduction of both cost and environmental impact of biocide treatments.

Over the years, different biofilm monitoring approaches have been proposed, based on the measurement of light scattering [7], turbidity [8], electrochemical impedance [9], vibration response of the monitored surface [10], diffusion limitation [11] and ultrasound [12] as indices of biofilm development. All the mentioned techniques cannot discriminate between biological and inorganic fouling, and this is a major problem, since these two different kinds of fouling require different treatments. Furthermore, the mentioned approaches have a low sensitivity, i.e. they cannot detect biofilm initial colonization phases, but only thicker bacterial layers; on the other hand, many biofilm related problems, such as MIC, start as soon as the first bacterial spots appear on a surface [13-15].

With the aim of overcoming the limitations presented by the existing sensors, a new electrochemical biofilm monitoring device (ALVIM) has been developed [16], exploiting the cathodic depolarization induced by biofilm growth on active-passive alloys (like Stainless Steels, Ti, Ni-Cu alloys, etc.) exposed to aerated waters. This phenomenon, which contributes to explain the higher corrosiveness of a natural water, in comparison with a sterile one, toward the mentioned alloys and less noble materials coupled with them, has been widely studied in the last 40 years [13, 17-20]. The electrochemical activity of natural aquatic biofilms was proven to be proportional to the percentage of surface covered by bacteria [21-22] therefore, measuring the biofilm electrochemical signal (BES), it is possible to know, on-line and in real time, which is the biofilm coverage on a given surface.

The effectiveness of ALVIM biofilm monitoring has been previously tested and demonstrated for seawater applications [16, 23]. The aim of this work was to evaluate the performances of this sensor for freshwater applications and, in particular, for paper mills. The work followed two subsequent steps:

(1) Preliminary sensor characterization, in a laboratory freshwater mesocosm, to study the response of this probe to biofilm growth in controlled conditions.

(2) Check of sensor response to biofilm growth in a paper mill, during ordinary working.

Materials and methods

Biofilm sensor

In order to monitor biofilm growth in the laboratory mesocosm and inside the water lines of the paper mill where the industrial testing has been carried out, the ALVIM biofilm monitoring system [16] was used. The phenomenon on which such a technology is based is the cathodic depolarization induced by biofilm growth on active-passive alloys exposed to natural aerated waters [13, 17-20]. In particular, as described in detail by Mollica *et al.* [21] and Faimali *et al.* [22-23], cathodic current density $i\,(E,t)$, measured at a given time t on a stainless steel (SS) or titanium (Ti) sample exposed to natural seawater and polarized at a fixed potential E, can be described by the relation:

$$i\,(E,t) = i_1(E) + [\, i_2(E) - i_1(E)] * \Theta\,(t) \qquad (1)$$

where $i_1(E)$ is the current density measured on the "clean" fraction of the SS/Ti surface and $i_2(E)$ is the one measured on the surface fraction $\Theta\,(t)$ [$0 \leq \Theta\,(t) \leq 1$] covered by biofilm. From equation 1, it

can be affirmed that, measuring potentials able to sustain a fixed cathodic current *i* during biofilm growth, it is possible to know when a specific biofilm coverage threshold, depending on the choice of imposed current, is reached.

The ALVIM probe (Fig. 1), already tested and validated both in laboratory and in other kinds of industrial environments [16, 24], is essentially a classic, conveniently simplified, three-electrode system, where the zinc counter-electrode (CE) plays also the role of pseudo-reference (RE). Connected to the zinc and to the titanium working electrode (WE), where biofilm growth is evaluated, there is an acquisition system, composed of three main parts: the first for substratum conditioning, the second for signal transduction and elaboration and the third for data transmission. The biofilm electrochemical signal (expressed as potential, in mV), measured in real-time, at chosen time intervals, is sent to a remote database, e.g. stored on a PLC/DCS or PC. In this case it was chosen to connect the ALVIM system to a PC, in order to simplify data transfer.

Since ALVIM is an electrochemical sensor, its signal can be influenced not only by biofilm, but also by some variations in the chemistry of the solution where the probe is immersed. For instance, biocide temporary injections causing pH decrease, or addition of oxidizing agents in the solution, are marked by peaks in the ALVIM signal, that add to the biofilm growth curve. Therefore, the overall graph indicates, on-line and in real time, if the biocide arrives properly at the point of the water line where the sensor is installed, and the biocide effect on biofilm.

The scientific principle and working modes of ALVIM sensor have been explained in depth by Pavanello et al. [16].

In the present work, biofilm threshold was set to 1% of WE (maximum sensitivity), to have an early-warning signal of biofilm growth.

Sensor preliminary characterization

Laboratory characterization of the biofilm sensor has been performed at CNR-ISMAR Genoa facilities, in a 20 L tank. The water in the tank was constantly renewed (1.5-2 L min^{-1}) with water pumped from a 150 L freshwater mesocosm, containing fishes and plants. In the water provided by this kind of mesocosm, biofilm covers a clean surface within some days after immersion [23].

Three ALVIM probes (replications) have been immersed in the experimental tank during three different periods (repetitions) of 11-16 days each, at a constant temperature of 24°C, in the dark, and BES was automatically acquired by a PC connected to the probes. After reaching the chosen biofilm coverage threshold value (set to 1%, i.e. maximum sensitivity), the probe sensitive surface (WE) was detached from the sensor to verify the real biofilm coverage on it. Between one test and the following one, the WE surface was cleaned and sterilized.

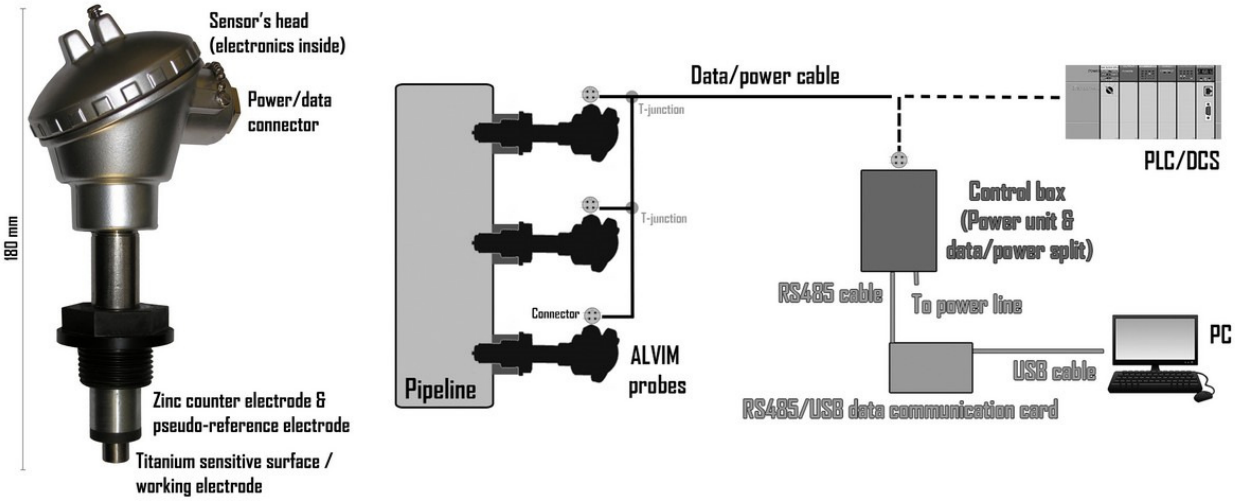

Fig. 1 - *ALVIM biofilm monitoring sensor (on the left) and scheme of a complete system (on the right).*

Biofilm coverage evaluation

WE surface area covered by bacteria was quantified by means of epifluorescence microscopy and software analysis. After the detachment from the ALVIM probe, the sensitive surface was gently rinsed in freshwater sterilized by filtration (Millipore, 0.22 µm pore size), in order to remove unattached cells, then it was fixed in 2% paraformaldehyde for 30 minutes, 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 [25]), samples were observed at 400× magnification using an Olympus BX41 epifluorescence microscope coupled with an UV filter block for DAPI. A digital camera CAMEDIA 5060 (Olympus) was used to acquire 30 images of 67500 μm^2 each, randomly chosen on the surface of the sample. Images were converted to tiff format (RGB color) and the surface fraction covered by bacteria was measured, on the 30 images, by means of "Image J" software; mean ± standard error (SE) was then calculated.

The paper mill

Italy is the fourth largest paper manufacturer in Europe [26], with about 9% of the total European production. The paper mill where this testing was conducted is located in the Province of Lucca (Italy), and belongs to one of the largest paper producers in Europe. This industrial plant produces tissue paper (paper towels, napkins, etc.), made both from virgin and recycled paper pulp. During the testing period, only virgin paper pulp was used. The production line of this paper mill had a water flow of approximately 800-900 m^3/h. During the testing period, process water temperature ranged between 26 and 30 °C.

The ALVIM probe was installed in the whitewater channel (i.e. the line that brings back process water after it has been used for transporting paper fibers) through a plug, at half of the height of the channel wall, to reduce the amount of possible deposit on probe surface. This location was chosen in order to monitor the water line section more distant from the biocide injection point, to have a "worst-case scenario" (i.e. where the chemical treatment was expected to be less effective). As for laboratory testing, biofilm coverage threshold value of the ALVIM sensor was set to 1%.

Water treatment in the paper mill

In order to avoid microbiological growth, a clean-in-place (CIP) treatment specific for paper mills was employed, including glutaraldehyde and isothiazolinone as active substances. These products were applied with "shock" treatments of one hour, six times a day. The application point was located at the beginning of the water line.

Biofilm monitoring trials in the paper mill

Since some maintenance activities had to be performed on the paper mill production line, between day 4 and day 14 after the installation of the biofilm monitoring system, the testing in the paper mill has been divided into three periods, in order to monitor biofilm growth dynamics and biocide treatment effectiveness in different conditions.

The first testing period (from day 1 to day 4) was employed to evaluate both ALVIM sensor functioning in the water line of the paper mill and the effectiveness of the biocide treatment usually applied in that industrial plant. Then the biofilm monitoring system was stopped, from day 4 to day 14. During this stop, the biocide application point was moved downstream in the water line. The sensor was left in place. During the second testing period, from day 14 to day 17, the effect of this change in the water treatment was evaluated with respect to biofilm growth.

On day 17, the biocide application point was moved back to its previous location. At the same time, the surface of the probe was cleaned with a pressurized water jet, in order to restart biofilm monitoring in conditions as close as possible to those of the first testing period.

Results and discussion

Sensor preliminary characterization

The testing carried out in laboratory (Fig. 2) showed a good correlation between sensor signal and real biofilm coverage. Bacterial growth on the sensor is indicated by the fast increase of the signal from the baseline (in this case between 300 and 450 mV) to the plateau (in this case between 700 and 750 mV). An increase of more than 100-150 mV in the signal, in the space of hours-days indicates biofilm growth.

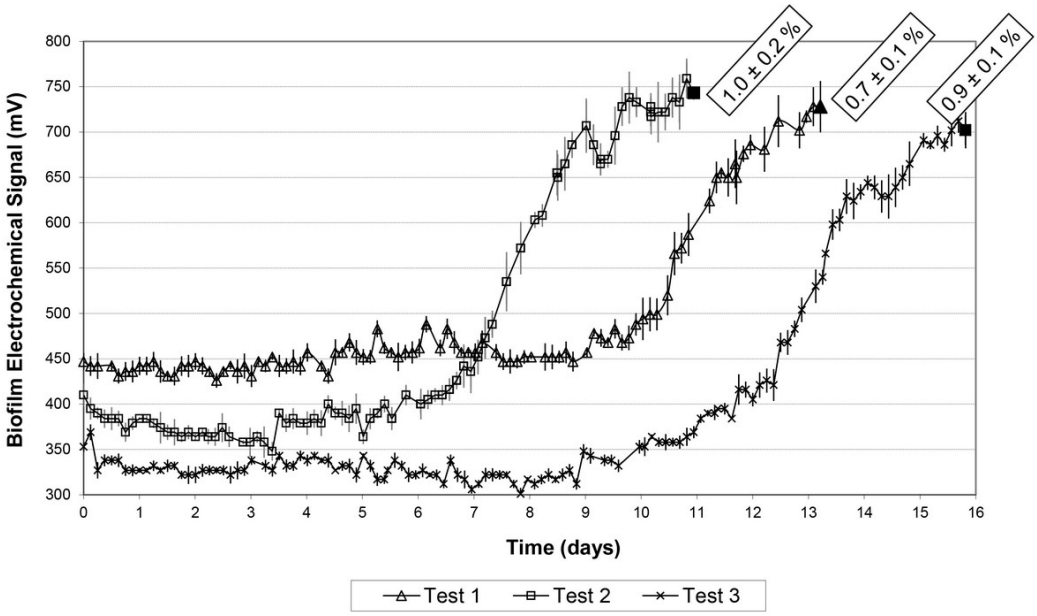

Fig. 2 - *Biofilm Electrochemical Signal (mean ± SE) during the three tests in the laboratory freshwater mesocosm. The real biofilm coverage at the end of each test, measured by epifluorescence microscopy, is indicated in the boxes (mean ± SE).*

The differences observed in the signal baseline values, which can be ascribed to different conditions of WE surface and mesocosm, do not influence biofilm monitoring, as explained by Eq. (1) and as already observed in seawater [16]. The different incubation times (7-12 days) observed in the three tests can be easily explained by variations in the biological activity of the mesocosm.

Biofilm monitoring trials in the paper mill

During the first testing period in the paper mill (Fig. 3), the ALVIM signal showed just a small increase over the baseline, indicating that biofilm growth inside the channel was not exceeding the chosen threshold (1% of surface area covered by bacteria). The peaks in the sensor signal (marked in Fig. 3 with small arrows) corresponded to the arrival of chemicals in the whitewater channel.

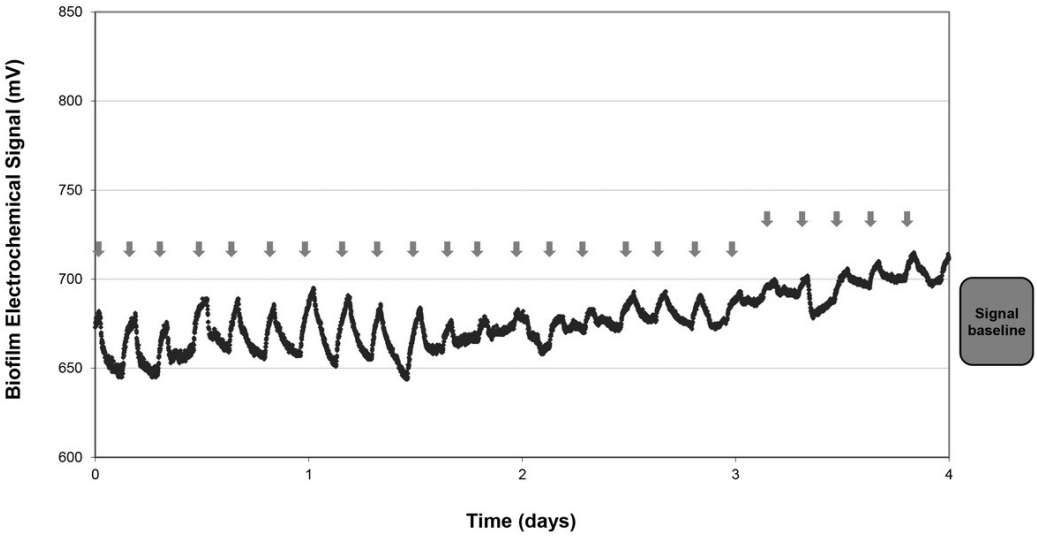

Fig. 3 - *Biofilm Electrochemical Signal during the first testing period. Grey arrows indicate biocide treatments.*

The applied biocides decreased the pH of the process water, and this influenced the ALVIM signal, as indicated by the peaks in the graph. The response of ALVIM signal to such treatment enabled the tracking of biocide injection. These data confirm that the applied treatment was appropriately distributed along the water line down to the point where the sensor was installed, and the treatment was effective in limiting biofilm growth even in this “worst-case scenario” (i.e. end of water line, far from biocide injection point).

After day 4, the biocide application point was moved downstream in the water line. At the start of the second testing period, the peaks in the ALVIM signal, indicating biocide treatments, were higher (Fig. 4, days 14-15). Since along the water line biocides are “consumed” (by organic matter, etc.), the longer is the distance between the application point and the measurement point, the lower is the residual concentration of such substances. This means that, after the change of application point, biocides were more concentrated in the section where the sensor was installed. However, on days 15-16 the height of biocide-related peaks decreased, indicating that a part of the injected chemicals was increasingly consumed before it reached the point where the sensor was installed. The change in the biocide application point implied also that the section of the line upstream to the new injection point was not sanitized anymore. These factors together can contribute to explain why, after just 2 days from ALVIM system restart, the sensor indicated that biofilm was growing inside the pipeline.

On day 17, biocide application point was moved back to its previous location, as indicated also by the height of the peaks in the ALVIM signal. At the same time, the surface of the probe was cleaned with a pressurized water jet (marked in Fig. 5 by the drop of signal on day 17). As can be observed in the graph (Fig. 5), biofilm growth started again in a very short time (1-2 days). Anyhow, the biocide treatment was able to keep microbiological growth under control; in fact, the biofilm electrochemical signal did not increase over 750 mV. Indeed, a complete biofilm development over the selected threshold causes an increase in the ALVIM signal up to 1200 mV [16, 23].

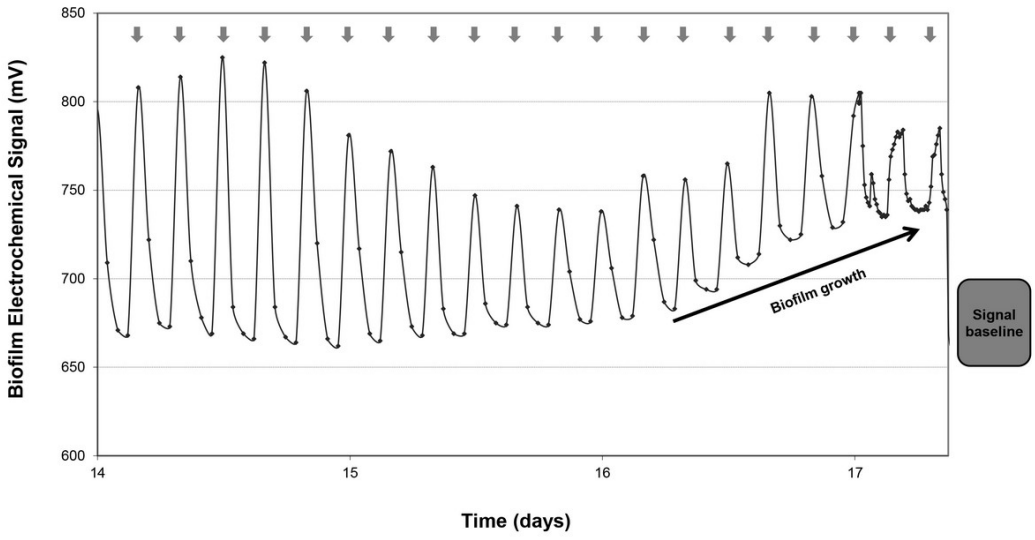

Fig. 4 - *Biofilm Electrochemical Signal during the second testing period. Grey arrows indicate biocide treatments.*

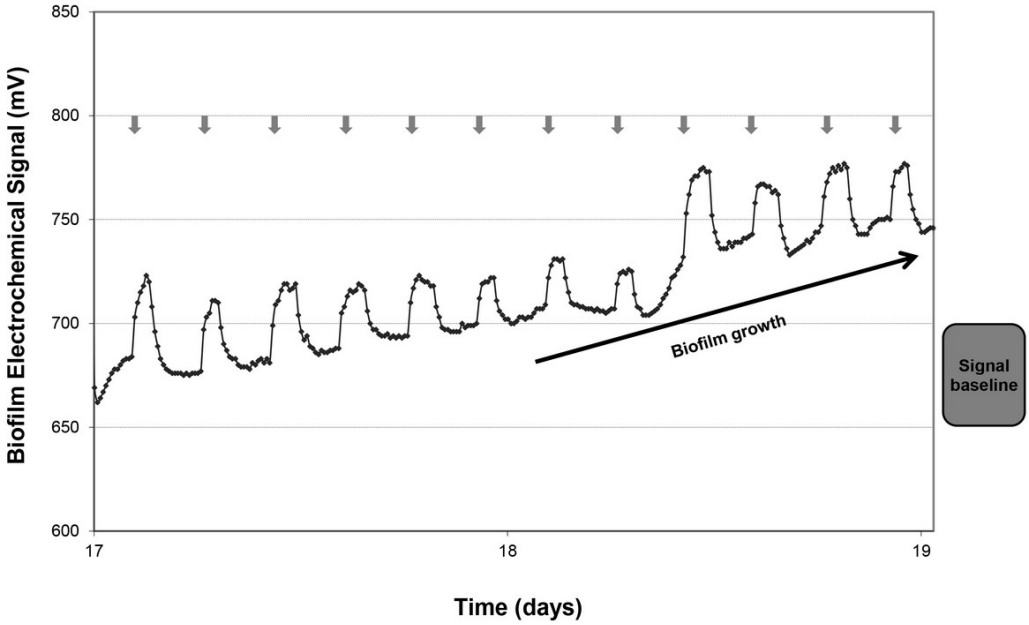

Fig. 5 - *Biofilm Electrochemical Signal during the third testing period. Grey arrows indicate biocide treatments.*

Compared to the standard laboratory analyses carried out in most paper mills, on-line biofilm sensors give the advantage of reducing the time and cost required for monitoring biological growth inside water lines. The devices available until now were mainly based on the measurement of parameters only indirectly related to biological activity (light scattering, turbidity, electrochemical impedance, vibration response of the monitored surface, diffusion limitation, ultrasound, etc., as previously discussed). These kinds of approach do not allow us to understand if the deposit forming inside pipelines is of biological origin or not. Thus, the indication given by those instruments cannot be used to decide if a biocide treatment is needed and, after its application, if such treatment has been effective.

In the preliminary characterization illustrated in this work, it has been demonstrated that the signal given by the ALVIM sensor is directly correlated to the bacterial coverage of the sensitive surface of the probe. The experimental data collected during the laboratory characterization confirm that, measuring potentials able to sustain a fixed cathodic current *i* during biofilm growth, it is possible to know when a specific biofilm coverage is reached, as per Eq. (1). Indeed, at the chosen sensitivity level (corresponding to a factory-set current density) the final potential measured by the probes corresponded to, approximately, 1% of sensitive surface covered by bacteria.

Applied to a real case study, this technique proved to be effective in monitoring both biofilm growth and the efficiency of cleaning treatments in different operative conditions.

Conclusions

Without an on-line monitoring system, it is hard for plant managers and technical staff to know exactly what happens inside the pipelines of their industrial facilities, and to evaluate the most appropriate actions to be taken. The experimental work carried out in this paper mill shows how, coupling standard biocide treatments with advanced on-line monitoring technologies, it is possible to keep track both of chemicals application and of its effectiveness toward eliminating biofilm in industrial water lines. The proposed approach could be profitably used to optimize biocide treatments, allowing the reduction of both their cost and environmental impact, at the same time increasing their efficacy and avoiding possible problems caused by microbiological growth.

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References

1. Terlizzi, A. and Faimali, M., "Fouling on artificial substrata". In: S. Dürr & J.C. Thomason (eds) Biofouling, Wiley-Blackwell, Oxford, pp. 170-184 (2010).
2. Flemming, H.C., Meier, M. and Schild, T., "Mini-review: microbial problems in paper production", Biofouling, 29:683-696 (2013).
3. Bajpai, P., Biotechnology for Pulp and Paper Processing, Springer, New York (2012).
4. Amann, R.I., Ludwig, W. and Schleifer, K.H., "Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation", Microbiology and Molecular Biology Reviews, 59:143-169 (1995).
5. Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M., "Microbial biofilms", Annual Review of Microbiology, 49:711-745 (1995).
6. Bjarnsholt, T., Kirketerp-Møller, K., Kristiansen, S., Phipps, R., Nielsen, A.K., Jensen, P.Ø., Høiby, N. and Givskov, M., "Silver against Pseudomonas aeruginosa biofilms", Apmis, 115:921-928 (2007).
7. Flemming, H.C., Tamachkiarowa, A., Klahre, J. and Schmitt, J., "Monitoring of fouling and biofouling in technical systems", Water Science and Technology, 38:291-298 (1998).
8. Klahre, J., Lustenberger, M. and Flemming, H.C., "Mikrobielle Probleme bei der Papierfabrikation. Teil III: Monitoring (Microbial problems in paper manufacturing. Part III: Monitoring)", Das Papier, 52:590-596 (1998).
9. Dheilly, A., Linossier, I., Darchen, A., Hadjiev, D., Corbel, C. and Alonso, V., "Monitoring of microbial adhesion and biofilm growth using electrochemical impedancemetry", Applied Microbiology Biotechnology, 79:157-164 (2008).
10. Pereira, A., Mendes, J. and Melo, L.F., "Using Nanovibrations to Monitor Biofouling", Biotechnology and Bioengineering, 99:1407-1415 (2008).
11. Foret, C., Merlet, N., Chaussec, G., Martemianov, S., Tribolet, B. and Hater, W. "Measurement of biofilm thickness: An effective Legionella risk assessment tool", http://www.pscleaner.gr/attachments/article/183/2010-02-bkgws_publication-measurement-of-biofilm-thickness%20(1).pdf. (Accessed 20 April 2016)
12. Maurício, R., Dias, C.J., Jubilado, N. and Santana, F., "Biofilm thickness measurement using an ultrasound method in a liquid phase", Environmental Monitoring and Assessment, 185:8125-8133 (2013).
13. Mollica, A. and Trevis, A., "Correlation entre la formation de la pellicule primaire et de la modification de la cathodique sur des aciers inoxydables experimentés en eau de mer aux vitesses 0.3 à 5.2m/s (Correlation between the formation of the primary film and the change in the cathodic reaction on stainless steels in seawater at speeds between 0.3 and 5.2m/s)", Proceedings of the Fourth Congress on Marine Corrosion and Fouling, 351-365 (1976).
14. Dexter, S.C. and La Fontaine, J.P., "Effect of natural marine biofilms on galvanic corrosion", Corrosion, 54:851-861 (1998).
15. Kimio, I., Matsuhashi, R., Toshiaki, K., Miki, O., Kihira, H., Watanabe, K. and Baker P, "Potential Ennoblement of Stainless Steel by Marine Biofilm and Microbial Consortia Analysis", Proceedings of Corrosion/2002, Paper No. 403 (2002).
16. Pavanello, G., Faimali, M., Pittore, M., Mollica, A., Mollica, A. and Mollica, A., "Exploiting a new electrochemical sensor for biofilm monitoring and water treatment optimization", Water Research, 45:1651-1658 (2011).
17. Scotto, V., Di Cintio, R. and Marcenaro, G., "The influence of marine aerobic microbial film on stainless steel corrosion behavior", Corrosion Science, 25:85-194 (1985).
18. Mattila, K., Carpen, L., Hakkarainen, T. and Salkinoja-Salonen, M.S., "Biofilm development during ennoblement of stainless steel in Baltic sea water: a microscopic study", International Biodeterioration and Biodegradation, 40:1-10 (1997).
19. Dulon, S., Parot, S., Delia, M.L. and Bergel, A., "Electroactive biofilms: new means for electrochemistry", Journal of Applied Electrochemistry, 37:173-179 (2007).
20. Little, B.J., Lee, J.S. and Ray, I.R., "The influence of marine biofilms on corrosion: a concise review", Electrochimica Acta, 54:2–7 (2008).
21. Mollica, A., Traverso, E. and Thierry, D., "On oxygen reduction depolarisation induced by biofilm growth on Stainless Steels in seawater", In: European Federation of Corrosion publications N°22, The Institute of Materials, London, pp 51-65 (1997).
22. Faimali, M., Chelossi, E., Garaventa, F., Corrà, C., Greco, G. and Mollica, A., Evolution of oxygen reduction current and biofilm on stainless steel cathodically polarized in natural aerated seawater", Electrochimica Acta, 54:148-153 (2008).
23. Faimali, M., Chelossi, E., Pavanello, G., Benedetti, A., Vandecandelaere, I., De Vos, P., Vandamme, P. and Mollica, A., "Electrochemical activity and bacterial diversity of natural marine biofilm in laboratory closed-systems", Bioelectrochemistry, 78:30-38 (2010).
24. Baldoni-Andrey, P., Lesage, N., Pedenaud, P. and Jacob, M., "Detection and Monitoring of Biofilm Growth in the Seawater Sulfate Removal Units", Proceedings of the SPE Produced Water Handling & Management Symposium, Paper No. SPE-174562-MS (2015).
25. Takata, K. and Hirano, H., "Use of fluorescein-phalloidin and DAPI as a counterstain for immunofluorescence microscopic studies with semithin frozen sections", Acta Histochemica et Cytochemica, 23:679–683 (1990).
26. Assocarta, "L'industria cartaria nel 2012", http://www.assocarta.it/en/documenti/category/2-chi-siamo.html?download=201%3Aindustria-cartaria-2012 (Accessed 20 April 2016).